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**Cytological, microspore culture, and genetic studies in the
interspecific backcross, *Brassica juncea* x *B. rapa* x *B. rapa*.**

by

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fulfillment of the requirement for the degree of Master of Science

in

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for the acceptance, a thesis entitled "Cytological, microspore culture, and genetic studies in the interspecific backcross, *Brassica juncea* x *B. rapa* x *B. rapa*." in partial fulfillment of the requirements for the degree of Master of Science in Plant Science.

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Abstract

Trait introgression from *Brassica juncea* to *B. rapa* was studied in an attempt to develop yellow-seeded, self-compatible *B. rapa* biotypes with acceptable oil and protein content, fatty acid profiles, and disease resistance. Microspore culture of *B. juncea/B. rapa* interspecific lines was unsuccessful in producing high frequencies of haploid embryos. An unbalanced genome or absence of a genotype-specific embryogenesis trait may have contributed to this failure. Molecular marker analysis verified the introgression of DNA from *B. juncea* to *B. rapa*, while cytological examination revealed the regular formation of 10 bivalent chromosome associations during meiosis. The interspecific lines had moderate to high levels of resistance to *Leptosphaeria maculans*, were susceptible to race 7v of *Albugo candida*, and segregated for resistance to race 2a of *A. candida*. Genetic variation in the BC_2F_3 *B. juncea/B. rapa* interspecific lines would allow selection for increased oil and protein content, a more nutritionally balanced fatty acid profile, and reduced days-to-maturity.

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% Percentage
μmol m^{-2}s^{-1} Light intensity in micromoles per meter squared second
°C Degree(s) Celsius
± Plus-minus
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Σ Summation

$1_{IV} + 9_{II} + 6_{I}$ 1 tetravalent, 9 bivalents, and 6 univalents (28 chromosomes total)
20-20-20 Nitrogen to phosphorus to potassium ratio
2n Diploid genomic chromosome number
4n Tetraploid genomic chromosome number

B_5 Supplemented microspore culture media
BC_1 First backcross generation
BC_1F_1 First backcross, first filial generation
BC_xF_y x^{th} backcross, y^{th} filial generation
bp Basepair

cm Centimeter
cv. Cultivar

ddH$_2$O Double distilled water
DH Doubled haploid(y)
DH_1 First generation doubled haploid progeny
DH_2 Second generation doubled haploid progeny
DNA Deoxyribonucleic acid
DSI Disease severity index

F_1 First filial generation
F_6 Sixth generation progeny
F_8 Eighth generation progeny

g Gram

kb Kilobasepair

L Liter

m Meter
MC Microspore culture
mg/l Milligram per liter
ml Milliliter
mm Millimeter

n Haploid gametic chromosome number
NIR Near infrared spectroscopy
NLN Nitsch and Nitsch liquid media
nm Nanometer

P₁ First parental generation
P_1 First parental generation
PCR Polymerase chain reaction
PMC Pollen mother cell

RAPD Random amplification of polymorphic deoxyribonucleic acid
RFLP Restriction fragment length polymorphism
rpm Revolutions per minute

S-**allele** Self-incompatibility allele
S-**locus** Self-incompatibility locus
SC Self-compatible
SI Self-incompatible
SI→SC Self-incompatible to self-compatible alteration
*S***LSGs** *S*-locus-specific glycoproteins
ssp. Subspecies
syn. Synonym

UV Ultraviolet

v/v Volume per volume
vs. Versus

w/v Weight per volume
WCC/RRC Western Canadian Canola/ Rapeseed Recommending Committee
WINISI Windows software (Infrasoft International LLC.)

x Ancestral haploid genomic chromosome number

Chapter 1

Introduction and literature review.

1.1 Introduction

High oil, protein-rich canola seeds containing low levels of saturated fatty acids and high levels of monounsaturated fatty acids contribute to the edible food market worldwide. The introduction of hybrid cultivars and genetically engineered varieties has sparked an interest in the general public and among producers with the promise of dramatic yield increases, greatly improved agronomic traits, e.g. disease resistance, and creation of niche markets. An alternative approach for trait improvement is through interspecific hybridization of oilseed canola species to each other, their wild allies, and distantly related relatives. Through this means, agronomic, physiological, and quality trait improvement through gene or chromosome introgression is possible.

Until quite recently, *Brassica juncea* Czern. and Coss. varieties grown as condiment crops in the Canadian prairies contained a high level of glucosinolates (approximately 200 micromoles per gram) and approximately 25% erucic acid (Woods *et al.* 1991). Canola quality oilseeds by statute must contain no more than 2% erucic acid and no more than 30 micromoles per gram of aliphatic glucosinolates in the oil-free meal (Seeds Regulations of the Canadian Seeds Act 1989). Currently, *B. juncea* canola quality cultivars with an oil content and fatty acid profile similar to *Brassica napus* L. are being developed for commercial release. *B. juncea* is highly resistant to blackleg disease and pod shattering. The superior heat and drought tolerance of *B. juncea* could enable it to be grown in dry areas of the southern prairies on an additional 4-6 million acres per annum (Stringam personal communication).

Brassica rapa L., almost exclusively a self-incompatible species, when inbred, suffers inbreeding depression (a loss in vigor and production). Selection and reselection of plants from large populations over many generations is necessary when dealing with self-incompatible species. This labor intensive process accounts for the marginal advances and subsequent yield plateau indicative of *B. rapa* breeding over the period of 1985 to the present day (Stringam personal communication). The existence of self-compatibility in *B. rapa* would enable plant breeders to use pedigree, single seed descent, and doubled haploid breeding methods in place of the current methods of mass and recurrent selection.

Introgression of traits from *B. juncea* to *B. rapa* through interspecific hybridization could improve the low level of resistance to blackleg disease in *B. rapa* through transfer of the excellent resistance in *B. juncea*. Currently, *B. juncea* is susceptible to race 2 of *Albugo candida,* while *B. rapa* contains high levels of resistance to race 2, but low-to-moderate resistance to race 7.

All heritable plant characteristics, including important agronomic and quality traits, are controlled by alleles (i. e. variant types of a gene) expressed at a single gene locus (qualitative inheritance) or 2 or more gene loci (quantitative inheritance). Regardless of the number of genes involved in the inheritance of a particular character trait, the presence of two different (i. e. heterozygous) alleles at a single genetic locus results in segregation of the expressed form of the trait in the progeny. A hypothetical example of this would be segregation of brown to yellow seedcoat color in a 3:1 ratio following self-pollination of a brown-seeded parent carrying both the dominant, brown allele and the recessive, yellow allele. In this scenario yellow seeds would only be

obtained if all of the loci controlling the seedcoat color trait were recessive. As conventional breeding methods rely on self-pollination to fix a certain trait, or a combination of traits, present in a segregating population, this process requiring 5 or 6 generations of inbreeding can be shortened to a single generation using microspore culture (Stringam personal communication). The intent of microspore culture, performed on an individual plant derived from a single cross, is the creation of thousands of unique, recombined doubled haploid embryos theoretically homozygous at all genetic loci. The Canadian canola cultivar, Quantum, was developed using the doubled haploid breeding method to provide resistance to the blackleg disease of canola in *B. napus* (Stringam *et al*. 1995a). Transfer of blackleg resistance from the Australian *B. napus* cultivar Maluka into susceptible breeding lines was accomplished in four years. The rapid development of Quantum using microspore culture gave rise to a high yielding canola variety with strong lodging and blackleg resistance (Stringam *et al*. 1995b). Thiagarajah *et al*. (1994) have recognized the utility of this technique for improvement of genic and chromosomal stability in *B. juncea/B. napus* interspecific hybrids.

Following interspecific hybridization between plants containing different chromosomes and/or genomes, the crossing event should be verified and the nature of the chromosomal interactions observed. Cytological examinations of meiotic chromosome spreads can be used to elucidate the chromosomal/genomic state of selected plants. Informative criteria include the number of chromosomes present, their physical associations (i. e. univalent, bivalent, or multivalent), and their genetic composition (*B. juncea*, *B. rapa*, or a *B. juncea-B. rapa* chimeric). The presence of the A-genome in both *B. juncea* (AABB, 2n=36) and *B. rapa* (AA, 2n=20) will lead to chromosomal

pairing between these species upon interspecific hybridization. As natural amphidiploid *Brassica* species (i. e. *B. carinata, B. juncea,* and *B. napus*) are secondary balanced polyploids, chromosomes from each diploid genome present will pair and assort independently during meiosis (Catcheside 1934, Alam 1936, Haga 1938). Therefore, the *B. juncea* and *B. rapa* A-genomes belong to similar, yet evolutionarily distinct chromosome sets of the *Brassica* n=10 cytodeme. The affinity between near-homologous chromosomes allowing for their preferential pairing is a function of the extent of conservation in their genetic codes after divergence from a common ancestor. Recombination between homologous chromosomes and homeologous regions of non-homologous chromosomes during meiosis both rely on sequence similarity and conservation in homology.

Molecular marker technology can be used to verify the occurrence of an interspecific hybridization event between both closely and distantly related species. The random amplification of polymorphic deoxyribonucleic acid-polymerase chain reaction (RAPD-PCR) assay is able to detect polymorphisms between the chromosomal sequences and/or genes from differing genomes. Genome-specific RAPD markers have been used to show that DNA was present from both formative species in *B. juncea/B. napus*-derived lines (Thiagarajah *et al.* 1994).

1.2 Interspecific hybridization in the *Brassicaceae*

The production of hybrid progeny from crosses of related species within a genus can be performed for several reasons. Briggs and Knowles (1967) identified four uses of interspecific hybridization: i.) to transfer one or a few genes from one species to another, ii.) to achieve new character expression not found in either parent, iii.) to produce new alloploid species, and iv.) to determine the relationship of one species to another. The use of interspecific hybridization to transfer genetic material, and the traits encoded therein, relies on the pairing of homologous chromosomes or pairing between homeologous regions of non-homologous chromosomes. The resultant recombinant chromosomes, containing DNA segments from both parental species of the hybrid, are incorporated as a heritable component of future progeny. With production of interspecific hybrids, trait transfer between *Brassica juncea*, *B. napus*, and *B. rapa* is possible because these species share a common genome. The genomic relationships among commercially important *Brassica* species (Fig. 1.1) were elucidated through numerous interspecific hybridization studies (Karpechenko 1922, Morinaga 1929abc, 1934, U 1935).

B. rapa (2n=20), *Brassica nigra* (2n=16), and *Brassica oleracea* (2n=18) are diploids with their differing haploid genomes designated A, B, and C, respectively (Morinaga 1934). The amphidiploid, or allotetraploid, species include *B. napus* (2n=38), *B. juncea* (2n=36), and *Brassica carinata* (2n=34) with genome designations of AB for *B. juncea*, AC for *B. napus*, and BC for *B. carinata* (Fig. 1.1). The common A-genome can serve as a bridge for the introgression of traits present on near homologous chromosomes through interspecific hybridization.

Figure 1.1   Genomic relationships between commercially important species of family *Brassicaceae* (Modified from: U 1935).

Brassica oleracea
CC (2n=18)

Brassica napus
AACC (2n=38)

Brassica carinata
BBCC (2n=34)

Brassica rapa
AA (2n=20)

Brassica juncea
AABB(2n=36)

Brassica nigra
BB (2n=16)

1.3 Microspore culture

In vitro culture of immature haploid microspores will cause a shift from the reproductive pathway (i. e. viable pollen formation) to the production of embryoids with a haploid genome complement. Nitsch and Nitsch (1969) demonstrated the importance of culturing anthers of *Nicotiana* species at a specific stage to stimulate the shift from pollen to embryo development. In *Brassica* species, the late uninucleate microspore stage was observed to be the most responsive to culturing (Keller *et al.* 1983, Kott *et al.* 1988). *In vitro* haploid microspore-derived embryos were first observed in *Datura innoxia* (Guha and Maheshwari 1964), while the first haploid plants developed using the microspore culture technique were obtained in *Nicotiana tabacum* (Bourgin and Nitsch 1967).

The primary function of the microspore culture/doubled haploid technique is the fixation of all genetically determined traits in the homozygous diploid state (Henderson and Pauls 1992). This technique allows the plant breeder to rapidly combine recessive or multiple traits in a single genotype. Conventional breeding methods rely on self-

pollination to fix a certain trait, or a combination of traits, present in a segregating population. This process requiring 5 or 6 generations of inbreeding can be shortened to a single generation using microspore culture (Stringam personal communication).

1.4 Self-incompatibility

In the flowering plants incompatibility systems function as a physiological barrier between self-pollination and self-fertilization to promote outcrossing and increase genetic variability. East (1929) described 'self-sterility' as the condition where "[v]arious species of hermaphroditic animals and plants exist in which a union between the male and female gametes produced by a given individual is difficult or even impossible, although each type of gamete is functional in other unions." 'Self-incompatibility' as coined by Stout (1917) has become the excepted designation for the genetically controlled process of recognition and rejection of otherwise viable self-pollen on the plant of origin. Mechanical barriers to self-pollination such as protandry, protogyny, monoecy, and dioecy rely on spatial and temporal patterns of pollen shed, stigma receptivity, and floral structure, while self-incompatibility is the result of chemical inhibition of self-fertilization (Bateman 1952).

The acquisition of incompatibility systems in higher plants can be seen as a response to selection pressure for optimum outbreeding. The sporophytic incompatibility system of the *Brassicaceae* is thought to have evolved from the gametophytic incompatibility system primarily present in monocotyledonous species (de Nettancourt 1977). The sporophytic determination of the pollen and pistil phenotypes, the recognition of self-pollen on the stigma surface, and the cellular interaction of glycoproteins present

in the pollen exine and the papillar cells of the stigma are the main characteristics of the *Brassica* incompatibility system. *Iberis amara*, an incompatible species, provided the first evidence of single locus multiallelic sporophytic control of both the pollen and style in the *Brassicaceae* (Bateman 1954). The self-incompatibility locus is known as the *S*-locus and the several distinct *S*-alleles code for specific *S*-locus specific glycoproteins (*SLSGs*). Independence, dominance, and mutual weakening relationships between *S*-alleles have been observed in *Iberis amara* (Bateman 1954). Dominance relationships between alleles in heterozygous genotypes were found to be non-linear with the possibility of different relationships (i. e. ranking) between pairs of alleles in the pollen and the style.

In artificial sterile *B. napus* the self-incompatibility loci of both the *B. rapa* (*syn. B. campestris*) and *B. oleracea* parental genomes are expressed (Hodgkin 1986), while SC *B. napus* lacks *S*-gene activity. When the self-incompatibility trait was introgressed into self-compatible *B. napus* from turnip, *B. campestris* ssp. *rapifera*, the *S*-alleles of *B. rapa* (*syn. B. campestris*) became active and an SC→SI alteration occurred (MacKay 1977). Lewis (1990) successfully introgressed the self-incompatibility trait into *B. napus* from the self-incompatible species *B. rapa* (*syn. B. campestris*) and *B. napus* ssp. *rapifera* (Swedes), but could not introgress the trait into the self-compatible amphidiploid using SI *B. oleracea*. The functioning of the introgressed *S*-alleles in the previously self-compatible *B. napus* suggests that amphidiploid *Brassica* species are self-compatible because of genetic disturbances between their two genomes and not as a result of deletions of the *S*-loci.

1.5 Cytology

Following interspecific hybridization, the interaction of chromosomes and/or genomes originating from the parental species can be observed during meiosis in the interspecific hybrid progeny. Identification of chromosomes from different *Brassica* genomes following an interspecific cross is difficult because of small chromosome size, the highly duplicated nature of the haploid elemental genomes (i. e. n = 8, 9, or 10), and the lack of a differential staining procedure for *Brassica* chromosomes (Barcikowska *et al.* 1995). The frequency of paired chromosomes undergoing recombination in interspecific hybrids can be used to estimate the degree of homology, i. e. the degree of evolutionary relatedness, between the parental species. The frequent formation of bivalents and multivalent associations were regarded as indicative of the degree of homology between the chromosomes of the widely related parents in the intergenomic cross of *B. juncea* and *Moricandia arvensis* (L.) DC, a possible donor of low photorespiration activity to the *Brassica* species (Bijral *et al.* 1995).

The presence of chromosome bridges, arising from inefficient resolution of crossover junctions between homeologous regions in an inverted orientation on non-homologous chromosomes, indicates the actual homology between chromosomes belonging to related genomes (Attia and Röbbelen 1986). The bridges formed when non-homologous chromosomes recombine, in a di- or trigenomic plant, are the result of either random synapses, homeologous pairing between non-homologous chromosomes, or crossing-over between the highly duplicated chromosome sequences common to the diploid genomes present (Riley and Kempanna 1963; wheat – *Triticum aestivum* – 2n=6x=42). The abnormalities imparted by these bridges have the ability to interrupt

normal meiotic behavior to a degree in which complete sterility of the hybrid results.

With the introgression of a chromosome segment or an entire chromosome into a recipient genome from a foreign donor, a situation arises where any newly introduced genetic material may have homologous counterparts present on chromosomes of the host genome. These complementary sequences on native or introduced chromosomes in the interspecific hybrids may result in the formation of multivalent chromosome associations. The presence of multi- and univalents, and their associated bridge-fragments and laggards, respectively, are meiotic abnormalities observed in *Brassica* interspecific hybrids (Kalasa-Balicka 1985; *B. juncea/B. carinata* interspecific hybrids). Multivalents have been observed, if only rarely, during diakinesis or metaphase I of meiosis in the 28-chromosome pollen mother cells (PMCs) of *B. juncea* x *B. rapa* interspecific hybrids (Sikka 1940: one out of 80 PMCs with $1_{IV} + 9_{II} + 6_{I}$). Although infrequent, this result confirms the pairing ability between the homeologous regions on the non-homologous chromosomes of the *Brassica* elemental genomes.

1.6 Molecular markers

Species-specific *Brassica* chromosome markers can detect DNA marker polymorphisms between the parental species in *Brassica* interspecific hybrids. Struss *et al.* (1995) found polymorphisms between *B. napus* and *B. nigra* for 252 out of 280 RAPD or RFLP (i. e. Restriction fragment length polymorphism) markers. The oligonucleotide primers used in the RAPD-PCR procedure can detect polymorphisms between parental genetic backgrounds in inter- and intraspecific hybrids. Thiagarajah *et al.* (1994) used genome-specific RAPD markers to indicate the presence of DNA from both species in

B. juncea/B. napus-derived interspecific hybrids. Quiros *et al.* (1991) found primers specific to the A-, B-, and C-genomes in both diploid and artificial amphidiploid *Brassica* species. Although the primers are initially random oligonucleotide sequences, their ability to detect polymorphisms between closely related species makes them useful as molecular markers following interspecific trait introgression. Using *B. napus-nigra* chromosome addition lines, 11 of 37 B-genome-specific primers discovered were mapped to four different *B. nigra* chromosomes (Quiros *et al.* 1991).

1.7 Quality analysis

1.7.1 Oil content and quality

Brassica varieties grown as a source of edible oil for use as margarine or cooking oil contain approximately 35-44% oil on a dry seed basis (Downey and Rimmer 1993). The relative proportion of fatty acids in the oil have been altered to meet demands for a desirable saturated to unsaturated fat ratio and the reduction of compounds with detrimental or disease-related effects.

Early breeding objectives of rapeseed focused on reduction of erucic acid and total glucosinolates. Originally, rapeseed contained high levels of erucic acid and was primarily used as a slip agent for tanks during World War II (Kumar and Tsunoda 1980). The implementation of the 'double low' standard (low erucic acid and glucosinolate content) led to the creation of a new edible oil crop. The next step was the alteration of the fatty acid profile to produce a nutritionally acceptable product. Lowered amounts of linolenic acid (C18:3) will improve storage characteristics of the oil as oxidation of the double bonds results in a rancid odor. Linoleic acid (C18:2), or Vitamin F, decreases

blood lipid cholesterol levels and reduces the risk of atherosclerosis. Canola oil contains approximately 61% oleic acid (C18:1), 22% linoleic acid (C18:2), 11% linolenic acid (C18:3), and 7% saturated fat (C16:0 + C18:0 + C20:0 + C22:0). These high unsaturated, low saturated, and moderate polyunsaturated fatty acid levels make canola oil one of the most nutritionally-balanced edible vegetable oils available for human consumption (Uppström 1995). The biochemical pathway of the nutritionally important fatty acids illustrates the relationship between the 18-carbon unsaturated fatty acids (Fig 1.2).

Edible oil quality goals from the late 1980s still being explored include the selection for lower erucic acid levels and attempts to reduce the amount of linolenic acid from 8-10% to less than 3%, while maintaining or increasing the level of linoleic acid (Downey and Röbbelen 1989). The occurrence of a strong negative correlation between the inheritance of linoleic and linolenic acid seriously hinders attempts to select for a high C18:2/C18:3 ratio (Röbbelen and Thies 1980a). The advent of genetic engineering has renewed research efforts into overcoming the theoretical erucic acid limit of 66.6% in the *Brassicaceae* and the creation of novel fatty acid profiles.

Figure 1.2   Biosynthetic pathway of major fatty acids (Jönsson 1977).

1.7.2 Protein content and quality

Following oil extraction the remaining portion of the seed can be used as a protein-rich feed source for livestock (Röbbelen and Thies 1980b). The oil-free meal is composed of cellulose and lignin (i. e. the hull or seedcoat), and protein fractions, with approximately 40% digestible protein being available (on a dry weight basis). The fibrous hull portion also contains protein, but the high cellulose content interferes with digestion in the stomach of non-ruminant animals.

The composition and thickness of the hull fraction determines seedcoat color in the *Brassica* species. Since the embryo is yellow, the yellow seed coat color is observed when the thickness of the hull portion is so reduced to be almost transparent, while black and brown seeds have thicker, pigment-containing seedcoats. Breeding for thinner seedcoats (i. e. yellow versus black and brown-seeded varieties) will reduce the fibre content and, ultimately, increase the protein content of the oil-free meal fraction of the seed (Stringam *et al.* 1974). The amino acid profile of the meal is well-balanced except for sub-optimal lysine levels.

1.7.3 Glucosinolates

The sulfur-rich glucosinolates present in the seeds, roots, leaves, and stems of the *Brassica* oilseed crops are responsible for their pungent odor and taste. After oil extraction, the well-balanced amino acid profile of the meal, composed of approximately 40% protein on a dry weight basis, is ideally suited as a feed supplement for beef, poultry, and swine (Röbbelen and Thies 1980b).

Solvent-based extraction of the oil fraction from intact seeds begins with physical

removal of the seedcoat. Prior to rupturing of cell walls the glucosinolates are intact and have no known physiological function (PBI Bulletin 1994). Mechanical processing allows myrosinase (i. e. thioglucoside glucohydrolase, E.C. 3.2.3.1), an extracellular enzyme, to physically interact with and enzymatically breakdown the glucosinolates into volatile compounds such as isocyanates, nitriles, and isothiocyanates. These toxic and goitrogenic compounds can cause growth and thyroid problems in poultry (Clandinin 1965) and swine (Bowland 1965), while their distinct mustard taste has been attributed to low palatability of the meal fraction by livestock (Bell and Devlin 1972).

In 1967, the *B. napus* cultivar Bronowski was found to contain a total glucosinolate content of approximately 12 micromoles per gram (on a whole seed basis), a significant reduction from the contemporary rapeseed average of 120 micromoles of glucosinolates per gram of oil-free meal (Krzymanski 1970, Lein 1970). Following the crossing of the low glucosinolate Bronowski line with a rapeseed variety containing less than 2% erucic acid, the first 'double low' (low erucic acid and glucosinolate content) *B. napus* variety, Tower, was released in Canada in 1973 (Stefansson and Kondra 1975). Today, breeders still rely on the 'Bronowski' genotype as a source of low glucosinolate germplasm (see Getinet *et al.* 1997). Currently, the accepted standards for canola quality oilseeds are no more than 2% erucic acid and no more than 30 micromoles per gram of aliphatic glucosinolates in the oil-free meal (Seeds Regulations of the Canadian Seeds Act 1989). Based on the guidelines of the Western Canadian Canola/Rapeseed Recommending Committee (WCC/RRC), cultivars currently being field tested in western Canada can contain no more than 0.5% erucic acid in the air-dried oil-free meal fraction and the seed must either contain no more than 12 micromoles of total glucosinolates per

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gram of whole seed at 8.5% moisture content or contain total glucosinolate levels no greater than those of the designated check, whichever is higher (WCC/RRC Procedures and Appendices 1997).

1.8 Agronomic traits

1.8.1 Days-to-maturity

The number of frost-free days required for a *Brassica* species to reach maturity is one of the major factors considered when determining the optimum growth zone for a particular crop. The short season regions of northern Alberta are best suited to *B. rapa*, which matures 10 days earlier than B. *napus* and *B. juncea* (Stringam personal communication). *B. napus* acreage is concentrated in, but not limited to, the central parkland regions of western Alberta, while the dry, hot southern region of the Alberta Prairies is usually seeded with drought and heat tolerant varieties of condiment *B. juncea*.

1.8.2 Seedcoat color

In the *Brassicaceae*, seed color is controlled by a minimum of 2 or 3 genes (Stringam 1980, Chen and Heneen 1992, Rashid *et al.* 1994, Sing and Aruna 1994) with *B. napus*, *B. rapa,* and *B. juncea* predominately expressing seedcoat colors of black, yellow-brown, and brown or yellow, respectively. Yellow seed color occurs naturally in varieties of *B. juncea* and *B. rapa*, but is absent in *B. napus* (Rashid *et al.* 1994). The thinner seedcoat found in yellow-seeded lines results in a greater quantity of oil and protein in the seed embryo (Stringam *et al.* 1974) as well as lower cellulose and fiber contents, clearer oil, and a higher feeding value than brown or black seeded varieties

(Tang *et al.* 1997). However, the embryo oil content does not actually differ between yellow and brown or black seeds (Rashid and Rakow 1995). Yellow seeds have a higher relative oil content because the embryo to seedcoat ratio is increased as a direct result of the absence of the thick, pigment-containing seedcoat layer of black and brown seeds.

1.9 Disease resistance

1.9.1 Blackleg disease

Blackleg disease of canola caused by *Leptosphaeria maculans* (Desmaz.) Ces. & de Not. (anamorph *Phoma lingam* (Tode:Fr) Desmaz.) is an important fungal disease in *Brassica* oilseed crops in Australia (Salisbury *et al.* 1995), Europe (West *et al.* 1999), and North America (Gugel and Petrie 1992, Pongam *et al.* 1999). Plant lodging and premature ripening, which account for the majority of seed yield loss caused by blackleg disease, are caused by stem-girdling necrotic cankers produced upon systemic infection of susceptible plants with *L. maculans*.

The use of genetically resistant cultivars is the most economical blackleg control strategy available (Rimmer and van den Berg 1992, Rempel and Hall 1996). Current techniques in biotechnology and molecular biology have been employed to introduce blackleg resistance genes from foreign sources into varieties adapted to the growth conditions of the Canadian prairies. Quantum, the first high yielding, high blackleg resistant *B. napus* variety released in Canada was produced using microspore culture technology (Stringam *et al.* 1995a). Blackleg resistance in Quantum was provided by a major gene or a closely linked set of genes inherited as a single complex from the Australian rapeseed cultivar Maluka (Stringam *et al.* 1992).

1.9.2 White rust disease

Albugo candida (Pers. Ex Hook.) Kuntze causes white rust disease in numerous cruciferous species. Susceptible *Brassica* plants develop white pustules on their inflorescences, leaves, and stems. Systemic spread of *A. candida* causes staghead formation, as a result of uncontrolled cell division and cell growth in individual buds or terminal bud clusters (Fan *et al.* 1983).

Pound and Williams (1963) assigned the biological variants of *A. candida* to distinct races based on their ability to infect a set of differential hosts. Race 2 is virulent on *B. juncea*, while *B. rapa* cultivars are susceptible to race 7 isolates. *B. napus* cultivars originating in Canada and Europe are resistant to all races of *A. candida* present in North America (Fan *et al.* 1983), but a few cases of susceptibility to race 7v have been reported in Chinese cultivars (Downey and Rakow 1987).

1.10 Objectives

The primary objective of the present research was the development of yellow-seeded, self-compatible *B. rapa* lines with acceptable oil and protein content, and resistance to white rust (races 2a and 7v) and blackleg diseases of canola (Stringam personal communication).

The self-incompatibility trait found in *B. rapa* poses a physiological barrier to self-fertilization. For this reason, *B. rapa* is an outcrossing species. In cases where self-pollination does occur, the resulting plants will suffer inbreeding depression. The introgression of the self-compatibility trait from *B. juncea*, through an intermediary *B. juncea/B. rapa* interspecific hybrid, will allow breeding strategies unique to self-

fertilizing, self-compatible species to be used in *B. rapa* breeding programs.

The *B. juncea/B. rapa* interspecific hybrids were formed by Dr. Gary Stringam prior to commencement of this research study. The bud pollination technique was used in the cross between a self-compatible F_8 *B. rapa* line with high oil and protein content and a *B. juncea* breeding line expressing good oil content, fertility, and yield potential, high resistance to blackleg, and susceptibility to race 2 of white rust (Stringam personal communication).

Microspore culturing of selected *B. juncea/B. rapa* interspecific hybrids is expected to fix the desirable traits through production of doubled haploid plants theoretically homozygous at all genetic loci. Conventional breeding methods rely on self-pollination for several generations, with reselection for traits after each step, to prevent segregation and promote fixation of the each trait within a single genotype.

Cytological examination and molecular marker work will be performed on all selected *B. juncea/B. rapa* interspecific hybrid lines to verify the original hybridization event and the presence of genetic material from both parental species. The elucidation of the genic/chromosomal state of the *B. juncea/B. rapa* interspecifics will provide information on the reproductive viability and stability of selected lines.

Introgression of important agronomic and quality traits from *B. juncea* to *B. rapa* through the *B. juncea/B. rapa* interspecific hybrids will be studied. The physiological maturity of *B. rapa* occurs 10 days earlier than *B.* juncea and B. *napus*. The addition of 3 or 4 days to the days-to-maturity index of *B. rapa* cultivars may impart a significant yield advantage (Stringam personal communication). Introgression of self-compatibility, the non-segregating yellow seedcoat color trait, the superior drought resistance, and the

excellent blackleg and white rust resistance of *B. juncea* into a *B. rapa* background with acceptable oil and protein content will be attempted.

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Chapter 2

Interspecific hybridization of Indian mustard (*Brassica juncea* L. Czern. and Coss.) and Polish rape (*Brassica rapa* L.) and microspore culture of BC_1F_1, BC_1F_2, BC_2F_1, and BC_2F_2 *B. juncea/B. rapa* interspecific hybrid lines.

2.1 Introduction

The introgression of traits across species and genus boundaries can be accomplished through interspecific and intergeneric hybridization, respectively. Within the *Brassicaceae*, numerous crosses have been performed amongst the diploid and amphidiploid species to successfully introgress traits across species boundaries, to study allelic variation and inheritance at important loci, and to provide a means to study and elucidate cytogenetic relationships among the *Brassica* species. Genetic resistance for white rust disease in *B. juncea* was strengthened with transfer of resistance from *Brassica carinata* (Singh and Singh 1987). The formation of *B. juncea/B. rapa* interspecific hybrids, from the cross: self-compatible (SC) *B. juncea* x self-incompatible (SI) *B. rapa*, would provide an opportunity for the introgression of the self-compatibility trait into *B. rapa* from *B. juncea* (current study). Anand (1981) used *B. rapa* (*syn. B. campestris)/B. napus* interspecific hybrids to show that alleles responsible for high erucic acid content in *B. napus* were expressed from the *B. oleracea* parental genome. *B. juncea* with low glucosinolate levels was selected from interspecific progeny of *B. rapa* x *B. juncea* crosses (Love *et al.* 1990). Barret *et al.* (1998) used RAPD analysis to localize a *B. juncea* blackleg resistance gene to a known linkage group, DY17, on a *B. napus* genetic map, following introgression of the gene into *B. napus*.

In *B. juncea*/*B. rapa* interspecific hybrids, near homologous chromosomes of the A-genomes of *B. juncea* (AABB, 2n=36) and *B. rapa* (AA, 2n=20) are expected to pair as homologous chromosomes during meiosis. At metaphase of the first meiotic stage, crossing over, i. e. genetic recombination, occurs at random between homologous or near homologous regions on adjacent chromosomes. The transfer of deoxyribonucleic acid (DNA) between the A-genome chromosomes within the *B. juncea*/*B. rapa* interspecific hybrid provides a bridge for trait introgression across species boundaries.

The direction of the interspecific cross between *B. juncea* and *B. rapa*, i. e. which parent will donate pollen and which will provide the ovules, influences the relative number of hybrid plants produced. Ramanujam and Srinivasachar (1943) and Mizushima (1950) performed the cross in both directions and found hybrids were more easily obtained when *B. juncea* was used as the female parent. When crossing an allopolyploid (*B. juncea*) and a diploid (*B. rapa*), Heyn (1977) suggested the use of the allopolyploid species as the female parent, especially if the two parents share a common genome. Sinskaia (1927) listed the reciprocal cross of *B. juncea* Czern. x *B. rapa* L. (*syn. B. campestris* L.) as "[s]pecies comparatively difficultly crossed with a small percentage of normally germinating seeds formed in result of these crosses, the average number of normal seeds per silique being less than one." Along with the low number of seeds per successful pollination, poor quality and thinness of seeds from this cross were noted.

The failure of viable seed formation in interspecific hybrids may be the result of either pre- or post-fertilization problems (Prakash and Hinata 1980). The inability of the pollen to germinate on the stigma and failure of the growing pollen tube to reach the

ovule are the two major pre-fertilization problems encountered when performing interspecific crosses. As a result, the formation of the zygote and production of viable seed will not occur. Some strategies used to avoid this problem are the addition of chemicals promoting germination and/or pollen tube elongation, bud pollination technique, radiation or high temperature treatment, temporal repetitions of pollinations, and stem or pistil grafting techniques (Reviewed by de Nettancourt 1977). A common problem found in most interspecific and intergeneric hybrids is the failure of the endosperm to develop normally because of a hormonal imbalance between the developing endosperm and maturing embryo (Prakash and Hinata 1980). Meng and Lu (1993) observed aborted seed in *B. napus/B. juncea* interspecific hybrid plants as a result of this post-fertilization barrier.

Microspore culturing of hybrid plants from both intravarietal and interspecific crosses can be used to fix desirable traits in doubled haploid progeny through enactment of homozygosity at all genetic loci (Henderson and Pauls 1992). The presence of chromosomal imbalances or unpaired chromosomes can cause fertility problems in interspecific hybrids. Thiagarajah *et al.* (1994) attributed the improved genic and chromosomal stability and increased fertility of doubled haploid *B. juncea/B. napus* interspecific hybrids to the microspore culture technique.

Specialized media is used to culture the microspores to initiate formation and promote development of high frequencies of well-formed embryos. Lichter (1981) developed a modified liquid medium, NLN, for use in *Brassica napus*. After surface sterilization of the buds, crushing and differential filtration, microspores released at the late uninucleate stage were cultured in NLN until embryos with a distinct root-shoot axis

and lateral cotyledons became visible to the naked eye. The presence of binucleate microspores, which had undergone the first pollen mitosis, resulted in a decreased efficiency of embryogenesis (Kott and Polsoni 1988). Centrifugation and replating of microspores in fresh NLN media increased embryo yields accordingly. Plating of the torpedo-shaped cotyledonary embryos on solid B_5 nutrient media (Appendix A) supplemented with gibberellic acid resulted in germination of *B. napus* embryos, and shoot initiation (Polsoni *et al.* 1988). Upon root establishment and emergence of the first true-leaf, a haploid plantlet was generated. Coventry *et al.* (1988) and Polsoni *et al.* (1988) provided overviews of the entire microspore culture technique in *B. napus* from bud selection and microspore harvesting through to plantlet formation, transplantation to soil, and colchicine treatment. Modifications to culture periods and conditions of the *B. napus* protocol (Coventry et al. 1988, Polsoni *et al.* 1988) led to microspore culture strategies amenable to both *B. juncea* (Thiagarajah and Stringam 1990) and *B. rapa* (Baillie *et al.* 1992, Burnett *et al.* 1992, Ferrie and Keller 1995, Guo and Pulli 1996).

Chromosome doubling of plantlets may occur during the microspore culture technique. These spontaneous doubled haploid plants are able to undergo meiosis, i. e. pollen and ovule formation, fertilization, and viable seed development. The percentage of spontaneous diploids observed by Coventry *et al.* (1988) varied from 10 to 30% for the *B. napus* genotypes cultured. Plants still in the haploid state can be treated with colchicine, a chromosome-doubling agent, to obtain diploid reproductive tissues able to produce functional gametes and, subsequently, doubled haploid, self-pollinated seed (Gland 1981). In this scenario a single doubled haploid cell is the end result of the colchicine treatment and a mosaic of diploid and haploid inflorescences on the

regenerated plant will be observed. The likelihood of obtaining at least one diploidized shoot on microspore-derived plants after colchicine treatment is approximately 60% (Gland 1981, Coventry *et al.* 1988). The level of ploidy in individual plants or single racemes has been reliably estimated in *B. napus* based on pollen-shed ability, pollen fertility, and seed setting behavior (Chuong *et al.* 1988b). Spontaneous diploids are able to directly produce pollen and set seed, while artificially doubled plants require several months to overcome the phytotoxicity of the colchicine treatment.

2.2 Materials and Methods

2.2.1 Interspecific hybridization

The *B. juncea* parent used to develop the F_1 interspecific hybrids was breeding line 95-1600 from the canola breeding program at the University of Alberta (Fig. 2.1). This line was derived from a modified backcross strategy involving early maturing *B. juncea cv.* Seeta (Patnagar dwarf), and 94-182-2, a *B. juncea* plant derived from an interspecific cross between 89-549-3, a low glucosinolate *B. juncea* selection, and 89-670-2, a *B. napus* selection with large pods. The *B. rapa* plant (97-313-2 [95-440-2]) used to develop the BC_1F_1 generation, and the BC_2F_1 *B. rapa* parents (97-12917 [95-442-3] and 97-12929 [95-439-2]; kindly provided by Mr. Tom Kubik) were self-compatible (dominant trait) F_8 sibling plants, from the interspecific cross of *B. rapa cv.* Eclipse x University of Alberta breeding line 83-52692B (an F_6 *B. napus* Dwarf line, with *B. napus cv.* Tower in its pedigree) (Fig. 2.1). These high oil, high protein lines expressed *B. rapa* phenotypic traits and had not been checked for embryogenic response.

Figure 2.1 *B. juncea/B. rapa* interspecific crossing scheme.

B. juncea cv. Seeta   University of Alberta
(Patnagar Dwarf)   breeding line 93-52170
(*B. juncea*)   (*B. juncea*)

94-182-2

94-644-1 x 94-522-1 [93-52170]   (*B. juncea/B. napus*)
(*B. juncea*) | (*B. juncea*)

94-1368-3 x 94-1372-2
(*B. juncea*) | (*B. juncea*)

95-755-4   x   95-757-1
(*B. juncea*)   (*B. juncea*)

Self-fertile *B. rapa cv.* Eclipse x
F_6 Dwarf line [*B. napus cv.* Tower]
90-5   90-25
(two plants from open-pollinated population)

several self-pollination generations

95-439, 95-440   95-442
(20-chromosome putative *B. rapa*)

P_1 95-1600-4 x 95-1603-3 [95-440-2]
(*B. juncea*) | (Fertile *B. rapa*)

F_1 97-54
(*B. juncea/B. rapa*)

97-54-1   x   95-313-2 [95-440-2]   97-54-2   x   95-313-2 [95-440-2]
(*B. juncea/B. rapa*) | (*B. rapa*)   (*B. juncea/B. rapa*) | (*B. rapa*)

BC_1F_1 97-2092-1 BC_1F_1 97-2094-1

BC_1F_2 x *B. rapa* BC_2F_1
97-2092-1 --> 98-751   97-12929 [95-439-2] x 97-2092-1 --> 98-954
97-2094-1 --> 98-959   97-12929 [95-439-2] x 97-2094-1 --> 98-955
                     97-12917 [95-442-3] x 97-2092-1 --> 98-956
                     97-12917 [95-442-3] x 97-2094-1 --> 98-957
                     97-2092-1 x 97-12929 [95-439-2] --> 98-958

BC_2F_2
Lines 98-2200 to 98-2253
(From individual plants from lines 98-954, 98-955, and 98-958)

BC_2F_3
Lines 97-24695 to 97-24718
(From individual plants from lines 98-2200 to 98-2253)

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The F_1 *B. juncea/B. rapa* hybrids were developed using the bud pollination technique (see below) by Dr. G. R. Stringam. Plants were grown in soil-free media (Stringam 1971) in a Conviron growth cabinet (Model PGR15) under a 16 hr photoperiod (with a minimum light intensity of 425 μmol m^{-2} s^{-1} of photosynthetically active radiation at the bench surface), with light/dark periods at 24 ± 2°C/ 21 ± 2°C. Plants were fertilized biweekly with 20-20-20 fertilizer. Sepals, petals, and anthers of unopened buds on the female parent were removed after opening of the first flower. Upon dehiscence of the pollen donor parent, viable pollen from select anthers was directly brushed onto the receptive stigmas. Reciprocal crosses were performed for each *B. juncea-B. rapa* crossing pair. Fertilized buds were covered for 4 days with 5 x 9 cm paper envelopes. Seed from each successful cross was collected and stored at room temperature.

Bud pollination was used to produce successive backcross generations and self-pollination steps were achieved through bagging of plants in 28 x 55 cm plastic bags (Cryovac Canada, Inc.) for the duration of flowering.

2.2.2 Microspore culture

B. juncea/B. rapa interspecific hybrid plants were grown with minimum stress in a Conviron growth cabinet (Model PGR15), under a 16 hour photoperiod (with a minimum light intensity of 425 μmol m^{-2} s^{-1} of photosynthetically active radiation at the bench surface), with light/dark periods at 20 ± 2°C/ 18 ± 2°C. Plants were fertilized biweekly with 20-20-20 fertilizer. Seed was directly sown in soil-free medium (Stringam 1971) or, when limited seed was available, germinated on sterile silica sand (Sil Silica Inc. Edmonton - Calgary, Alberta – Grade Sil. 1) in 60 x 15 mm Petri plates at room

temperature on the bench surface. Seedlings were transferred to six-inch pots 3-5 days after plating. Prior to bolting of the main stem the growth cabinet temperature parameters were changed to $10 \pm 2°C/ 5 \pm 2°C$ (light/dark). After opening of the first flower, clusters containing a wide size range of unopened buds were collected at approximately 10:00 am from the main and lateral racemes. The number of culture events required for a single collection was dependent on the number of plants sampled at a given time and the quantity of bud clusters per plant to be cultured. Individual plants were cultured for periods of up to 3 months. Individual culture events were comprised of 4 sub-samples, each composed of a maximum of 50 buds. To pinpoint the correct cytological stage, optimal bud size (i. e. petal/anther ratio) for each *B. juncea/B. rapa* interspecific hybrid line cultured was established through cytological staging of microspores. Bud sizes corresponding to petal/anther ratios of $1/3 - 3/4$ were used to approximate the late uninucleate stage of microspore development. Microspore culture protocols developed for *B. juncea* (Thiagarajah and Stringam 1990) and *B. rapa* (Baillie *et al.* 1992) were used to culture buds from each BC_1F_1 and BC_2F_1 plant. For the *B. rapa* protocol, culturing was performed immediately and, if necessary, four hours after collection. The *B. juncea* protocol required cold treatment of the buds in darkness at 4°C for 4-7 days. Surface sterilization in 7% sodium hypochlorite for 10 minutes and three rinses in double distilled water (ddH_2O) preceded crushing of the buds in 15 ml of cold B_5 wash (Appendix A) using a sterile mortar and pestle. Microspores were filtered through 64 and 44 μm nylon mesh and washed three times in 15 ml of B_5 wash following centrifugation at 1000 rpm for 5 minutes. After the final centrifugation step, the supernatant was removed and 1 ml of modified Lichter's medium (Lichter 1981), supplemented with 0.5

mg/l naphthaleneacetic acid and 0.05 mg/l N^6-benzyladenine, was added to a 50 ml Falcon tube for each bud sampled. Microspore concentration was determined with a haemocytometer for each individual line cultured to ensure the petal/anther criteria reliably estimated 75 000-100 000 microspores per milliliter of NLN (Coventry et al. 1988). The *B. juncea* protocol required samples containing microspores in NLN13 (i. e. NLN with 13% (w/v) sucrose) (Appendix B) to be separated as 10 ml aliquots into Petri plates (60 x 15 mm) and incubated in darkness at 35°C for 18 hours and then at 30°C for 10-14 days (Thiagarajah and Stringam 1990). The *B. rapa* protocol (Baillie *et al.* 1992) required centrifugation (1000 rpm for 5 minutes) and resuspension of microspores in fresh NLN media after 24 hours at 32°C in NLN17 (i. e. NLN supplemented with 17% (w/v) sucrose and 0.1 mg/l benzyladenine) (Appendix B). Immediately following the switch to fresh NLN10 media (i. e. NLN supplemented with 10% (w/v) sucrose and glutamine), plates were placed at 25°C in the dark for an additional 10-14 days. The cultures were then transferred to a gyratory shaker (set at 60 rpm) and maintained at room temperature under continuous light until the embryos turned green (*B. rapa* protocol) or in darkness for 4-10 days (*B. juncea* protocol). Throughout the entire culture period, most cultures produced either no embryos, or aborted embryos that appeared as tiny white spherical objects (visible without a magnifying device). In some cultures, callus-like masses of undifferentiated cells developed and eventually produced a shoot-root axis upon continuous subculturing (Keller and Armstrong 1981, Chuong *et al.* 1988b). Callus-like material with one true-leaf were transferred to sterile Magenta boxes containing solid Murashige and Skoog medium, with 2% sucrose and 1% agar, to promote root establishment and increase resiliency to stresses. Torpedo-shaped embryos, which

developed a distinct shoot-root axis and lateral cotyledons, were plated on B_5 solid media (Appendix C) and moved to a 4°C incubator, with an 8-hour photoperiod, for 10 days. After cold treatment, the plates were maintained at room temperature under Verilux© full spectrum fluorescent plant growth lights with a 16-hour photoperiod. Individual plantlets with at least one true-leaf and well-formed roots were then transferred to 32 plastic pots (6 x 6 cm) containing soil-free medium (Stringam 1971), covered with plastic containers for 2-4 days, and placed in a Conviron growth cabinet (Model PGR15) under a 16 hour photoperiod (with a minimum light intensity of 425 µmol m^{-2} s^{-1} of photosynthetically active radiation at the bench surface), with light/dark periods at $20 \pm 2°C/ 15 \pm 2°C$. When plants reached the 3- to 5-leaf stage they were transplanted to six-inch pots and transferred to a greenhouse with day/night temperatures of approximately 20°C/ 15°C, a 16 hour photoperiod, and natural light supplemented with high intensity sodium vapor lights (HID Sylvania, Canada) producing a light intensity of 425-450 µmol m^{-2} s^{-1} of photosynthetically active radiation at the bench surface. Plants were fertilized biweekly with 20-20-20 fertilizer. Spontaneous diploid plants were enclosed within 28 x 55 cm plastic bags (Cryovac Canada, Inc.) to limit cross-pollination, and harvested by hand when seed reached physiological maturity. Roots of haploid plants were thoroughly cleaned with water, leaves on the lower part of the stem were removed, and then the roots were immersed in a 3.4% (w/v) colchicine solution for two hours and twenty minutes. Treated plants were returned to the greenhouse under a stringent watering regime to encourage development of diploid racemes. Pollen-producing (i. e. diploidized) racemes were covered in plastic bags to limit cross-pollination, and seed was harvested by hand when physiological maturity was reached.

2.3 Results

2.3.1 Interspecific hybridization

The plants of five putative BC_1F_1 *B. juncea/B. rapa* interspecific hybrid lines were visually examined for species-specific phenotypic traits and features commonly observed in *Brassica* interspecific hybrids. Four plants (97-2091-1, 97-2092-1, 97-2094-1, and 97-2094-2) representing three of the lines were dubbed 'interspecific hybrids' based on phenotypic characteristics which were intermediary between the parents (i. e. leaf type, leaf clasping, and/or relative position of the bud cluster to the opened flowers) or represented physiological stresses imposed by the interspecific condition (i. e. decreased pod length/size and/or gametic sterility). Using these criteria, five plants from the other 2 lines (97-2090 and 97-2093) were most likely composed of a *B. juncea*, not a *B. juncea/B. rapa*, genetic background. These plants may have been formed through accidental contamination or pollination with a *B. juncea* pollen donor, or the spontaneous development of diploid eggs arising from abortion or failure of meiosis (diplospory), followed by formation of diploid embryos from these unreduced female gametes (parthogenesis) (Heslop-Harrison 1983).

Based on phenotypic traits, BC_1F_1 plant 97-2092-1 resembled the *B. juncea* parent, while 97-2094-1 expressed more *B. rapa*-type characteristics. These F_1-derived siblings were backcrossed for the second time with *B. rapa* to facilitate seed production and ensure the continued propagation of the interspecific hybrid lines (Fig. 2.1, Table 2.1). The average number of BC_2F_1 seeds formed per pod from the crosses in which *B. rapa* was the female plant were 0.87, 1.25, 2.08, and 2.18. The low number of seeds developed in each pod is indicative of the interspecific condition, but the low

percentage of pods formed per buds pollinated (32.3%) was expected as *Brassica* species normally produce many more flowers than pods. When the *B. juncea/B. rapa* interspecific hybrids served as female parent, the cross of 97-2092-1 x 9712929 was the only success with 5 seeds resulting from the 24 pods formed (Table 2.1). The *B. juncea* parent was expected to produce more viable seed when acting as the ovule donor in the reciprocal crosses performed between *B. juncea* and *B. rapa*. While F_1 and BC_1F_1 interspecific hybrid seed was formed in this direction, the second backcross generation was produced in the pods of the *B. rapa* parent.

Although cytological examination (i. e. chromosome counts) and species-specific DNA marker technology were used to validate the genomic composition of selected lines (See Chapter 3), the *Brassica* interspecific hybrids were originally verified using visual traits (see above) and seed appearance and seed yield. The seeds used to sow the BC_1F_1 and BC_2F_1 generations were thin and of poor quality, while all of the parental lines (except for plant 94-182-2) had spherical and well-formed seeds. The lack of replication in the field observation trial negated the statistical relevancy of the seed yield data, which could have been used to study the fertility, and, therefore, the self-compatibility trait in the BC_2F_2 *B. juncea/B. rapa* interspecific hybrid lines. However, when compared with the *B. juncea* and *B. rapa* parental lines, the seed yields of the BC_2F_1 and BC_2F_2 lines support the interspecific composition of the *B. juncea/B. rapa* interspecific hybrid lines (Table 2.2). The average BC_2F_1 and BC_2F_2 seed yields were less than one gram per plant except for the nine plants sown from the selfed seed of the BC_2F_1 line 98-958 (1.69 grams per plant). When grown in the greenhouse alongside the BC_2F_1 and BC_2F_2 plants, the *B. juncea* parental lines had average seed yields of 5.39, 1.42 and 0.05 grams (Table

2.2). The *B. rapa* backcross parent, 97-12929, produced 1.97 grams of seed upon selfing. Although parental lines produced more seed than interspecific hybrid lines, the small numbers of plants per line and lack of a replicate sample limited this finding to trend status, not a statistically significant finding. The poor seed yield (0.05 grams) of the single plant from *B. juncea* parent 94-182-2 may have failed to provide an accurate indication of the yield potential of this line.

2.3.2 Microspore culture

Embryos derived from the putative BC_1F_1 line 97-2093 were torpedo-shaped with well-formed cotyledons. Species-specific *Brassica* phenotypic characteristics (see Section 2.3.1) indicated that the plants of lines 97-2090 and 97-2093 were of a *B. juncea* genetic background. All embryos formed from the culturing of these lines were discarded. Embryos derived from the *B. juncea/B. rapa* BC_1F_1 (97-2092 and 97-2094), BC_1F_2 (98-751), BC_2F_1 (98-954, 98-955, and 98-958), and BC_2F_2 (98-2200 to 98-2253) interspecific hybrid lines were composed of callus-like masses of undifferentiated cells. Efficiency of embryo formation was based upon the number of culture events performed on individual plants, independent of microspore culture protocol (*B. juncea* or *B. rapa*). Although the two protocols used to culture the *B. juncea/B. rapa* microspores may have differed in their effectiveness to enact the switch from pollen to embryo formation, the sequence of treatments of microspores under both protocols were identical, except for slight modifications to incubation periods and media compositions. Since one milliliter of NLN was used to plate each bud cultured, the absolute number of buds sampled per culture event was irrelevant and each plate should have contained 75, 000 to 100, 000

microspores per milliliter of liquid media. Of the 105 individual culture events performed on the four BC_1F_1 plants only 7 of the cultures produced embryos, while culturing of the BC_2F_2 plants from lines 98-2200 to 98-2253 resulted in formation of 125 embryos from 14 separate cultures (Table 2.3). Plants from BC_1F_2 line 98-751 and BC_2F_1 lines 98-954, 98-955, and 98-958 yielded 1197 embryos from 67 successful culture events of 354 performed. Overall, 16.8% (52 of 309) of the *B. rapa* and 11.9% (36 of 302) of the *B. juncea* cultures produced at least one embryo. The efficiencies of the two protocols were similar, except for cultures performed on lines 98-954 to 98-958. The *B. rapa* microspore culture protocol performed on these lines accounted for 41 of the 88 successful cultures and 54.4% (728 of 1339) of the overall embryo production (Table 2.3).

The duration between culturing of immature microspores and transfer of embryos (i. e. callus masses) to cold incubation ranged from 12 to 48 days. For the 201 separate transfers of varying numbers of embryos to the 4°C incubator, the average delay was 23.8 days (data not shown). The extra time required for the greening of the callus masses was spent on the gyratory shaker, not in the initial (dark) incubation period. Upon removal from cold treatment, shoot initiation from callus masses was not synchronous for individual plates or between the replicated plates derived from a single culture event. The amount of time required to stimulate shoot initiation and formation of the first true-leaf varied from 1 or 2 weeks to 3 months (data not shown).

Table 2.1   Average number of BC_2F_1 seeds formed per pod from the reciprocal cross of BC_1F_1 interspecific hybrids (97-2092-1 and 97-2094-1) and self-fertile *B. rapa* (97-12917 and 97-12929). [ ] = the number within the brackets represents the parent used to produce the plant serving as the female or male parent. n/a = not applicable.

Female Parent	Male Parent	Seed Accession	# of buds	# of pods	# of seeds	# of seeds/pod
97-2092-1	97-12917 [95-442-3]	n/a	66	31	0	0
97-12917 [95-442-3]	97-2092-1	98-956	106	23	20	0.87
97-2092-1	97-12929 [95-439-2]	98-958	59	24	5	0.208
97-12929 [95-439-2]	97-2092-1	98-954	50	11	24	2.18
97-2094-1	97-12917 [95-442-3]	n/a	41	27	0	0
97-12917 [95-442-3]	97-2094-1	98-957	125	20	25	1.25
97-2094-1	97-12929 [95-439-2]	n/a	25	21	0	0
97-12929 [95-439-2]	97-2094-1	98-955	51	12	25	2.08

Table 2.2 Average greenhouse seed yield (in grams) of BC_2F_1 and BC_2F_2 *B. juncea/B. rapa* interspecific hybrid plants and *B. juncea* and *B. rapa* parental lines. [ ] = number within brackets represents the parent or cross used to produce the plant or line, respectively, from which the BC_2F_1 and BC_2F_2 progeny were derived, g = grams, n/a = not applicable.

Progeny of:	Species	Status of progeny	Number of progeny	Average Seed Yield (g)
97-12929 x 97-2092-1	*B. juncea/B. rapa*	BC_2F_1	2	0.6
97-12929 x 97-2094-1	*B. juncea/B. rapa*	BC_2F_1	1	0.7
97-12917 x 97-2092-1	*B. juncea/B. rapa*	BC_2F_1	2	0.585
97-12917 x 97-2094-1	*B. juncea/B. rapa*	BC_2F_1	2	0.675
Self-pollinated 98-954 [97-12929 x 97-2092-1]	*B. juncea/B. rapa*	BC_2F_2	38	0.596
Self-pollinated 98-955 [97-12929 x 97-2094-1]	*B. juncea/B. rapa*	BC_2F_2	44	0.61
Self-pollinated 98-956 [97-12917 x 97-2092-1]	*B. juncea/B. rapa*	BC_2F_2	7	0.613
Self-pollinated 98-957 [97-12917 x 97-2094-1]	*B. juncea/B. rapa*	BC_2F_2	66	0.951
Self-pollinated 98-958 [97-12929 x 97-2092-1]	*B. juncea/B. rapa*	BC_2F_2	9	1.69
Self-pollinated 94-182-2	*B. juncea*	Self-pollinated formative parent	1	0.05
Self-pollinated 94-644 [*cv.* Seeta (Patnagar dwarf)]	*B. juncea*	Self-pollinated formative parent	5	5.39
Self-pollinated 95-1600 [*cv.* Seeta x 94-182-2]	*B. juncea*	Self-pollinated P_1 parent	3	1.42
Self-pollinated 97-12929 [95-439-2]	*B. rapa*	Self-pollinated backcross parent	3	1.97

Table 2.3 Microspore culture and embryo production efficiencies for *B. juncea/B. rapa* interspecific hybrid plants sampled from BC_1F_1 (97-2091, 97-2092, and 97-2094), BC_1F_2 (98-751), BC_2F_1 (98-954, 98-955, and 98-958), and BC_2F_2 (98-2200 to 98-2253) lines. ( ) = number of successful cultures of total cultures attempted.

Lines	Number of plants	Successful *B. rapa* Cultures	Number of embryos	Successful *B. juncea* cultures	Number of embryos	Successful cultures (both protocols)	Number of embryos (total)
97-2091 97-2092 97-2094	4	3.92% (2 of 51)	7	9.26% (5 of 54)	10	6.67% (7 of 105)	17
98-954 to 98-958	61	23.2% (41 of 177)	728	14.7% (26 of 177)	469	18.9% (67 of 354)	1197
98-2200 to 98-2253	11	11.1% (9 of 81)	71	7.04% (5 of 71)	54	9.21% (14 of 152)	125
Totals	76	16.8% (52 of 309)	806	11.9% (36 of 302)	533	14.4% (88 of 611)	1339

Even after 2 months of subculturing the callus-like embryos derived from BC_1F_1 plants 97-2091-1, 97-2092-1, 97-2094-1, and 97-2094-1 and the BC_2F_2 plants from lines 98-2200 to 98-2253, neither a true-leaf nor plantlet was formed. From the embryos derived from BC_2F_1 lines 98-954, 98-955, and 98-958 and the BC_1F_2 line 98-751, a total of 108 plantlets were transferred to soil. Three of the plants were recognized as spontaneous diploids, based on pollen production, while only one of the 39 haploid plants treated with colchicine was able to produce seed. The colchicine-doubled plant was derived from BC_2F_1 plant 98-954-5, while the 3 spontaneous doubled haploid plants originated from BC_1F_2 plant 98-751-3 (one DH_1 plant) and BC_2F_1 plant 98-954-3 (two DH_1 plants). An insufficient amount of seed was available from these 4 DH_1 plants for 6-meter observation rows and the time required to obtain self-pollinated DH_2 seed could not be accommodated prior to sowing the 1999 field observation trial plot. These lines did not undergo a seed-increase generation and were not studied any further.

2.4 Discussion

2.4.1 Interspecific hybridization

Physical barriers to interspecific hybridization observed in crosses between *Brassica* species include the inhibition of pollen germination or pollen tube penetration of the stigma and the cessation of pollen tube growth in the various tissues of the stigma. Even a successful hybridization (i. e. fertilization) event produces only one or two poor quality, thin, shriveled seeds per pod, as observed after the reciprocal crossing of *B. juncea* and *B. rapa* (*syn. B. campestris*) (Sinskaia 1927). The *B. juncea* parent was used as the ovule donor in the original cross, while the *B. juncea/B. rapa* interspecific hybrids and the *B. rapa* parental lines were used as the female parents in the first and second backcross generations, respectively (Fig. 2.1). This strategy was based upon the observation that hybrid seed developed in greater numbers when *B. juncea* was used as the female parent (Ramanujam and Srinivasachar 1943, Mizushima 1950) and the suggested usage of the allopolyploid species as the female when crossing an allopolyploid (*B. juncea*) and a diploid (*B. rapa*) species sharing a common genome (Heyn 1977). Although interspecific plants produced limited seed during each of the backcross and self-pollination generations, nearly all of the well-formed, spherical seed germinated upon sowing (unpublished observation).

The non-homology of the divergent A- and B-genomes of *B. juncea* and *B. rapa*, and the genic and chromosomal imbalances resulting from this cross, can be identified as the major factor responsible for poor seed quality and low seed yield in the primary interspecific hybrids. The *B. rapa* parent, derived from an interspecific cross between *B. napus* and *B. rapa*, contained a dominant trait for self-compatibility (Stringam

personal communication). This allowed the canola breeding program at the University of Alberta to use the pedigree breeding strategy to select for F_7 lines (i. e. 95-439, 95-440, and 95-442) with *B. rapa* phenotypes, excellent vigor, and good quality traits. The fertility problems observed within these lines were attributed to residual heterozygosity for chromosomal abnormalities associated with the original interspecific cross, rather than inbreeding depression, since vigor and general agronomic performance of these lines was acceptable (Stringam personal communication). The reduced pollen and ovule fertility in the BC_2F_1 and BC_2F_2 *B. juncea/B. rapa* interspecific hybrid plants (Table 2.2) was probably a result of genic and chromosomal imbalances, and not a sporophytic incompatibility response, as the interspecific plants were vigorous and produced many pods (if not seed). Thiagarajah *et al.* (1994) noted ovule and pollen sterility in *B. juncea/B. napus* interspecific hybrid lines, which were developed for trait introgression between these species. Irregular chromosome and/or genome associations during meiosis in pollen mother cells (PMCs) of interspecific hybrid plants were found to be strongly correlated with observed sterility problems. Since doubled haploid embryos homozygous for chromosomal imbalances or abnormalities should be aborted as a result of the problems associated with chromosome duplications or deficiencies, the MC technique was expected to restore fertility in the *B. juncea/B. napus* interspecific lines. Fertility problems in interspecific hybrids were also noted in several other crosses within the *Brassicaceae* (Roy 1977, Prakash and Chopra 1990).

2.4.2 Microspore culture

Although the *B. juncea/B. rapa* germplasm was amenable to microspore culture, the majority of embryos formed consisted of callus-like masses of undifferentiated cells that required continuous subculturing before a shoot-root axis was formed. Torpedo-shaped embryos, which have the ability to produce significantly higher frequencies of regenerated plants than other embryo types (Chuong and Beversdorf 1985), were only produced in low frequencies. Several factors may have contributed to the poor torpedo-shaped embryo yields. These include the lack of a gene conditioning embryogenesis, the occurrence of genotype, plant age or sampling dependent responses to culturing, or the effects on embryo yield and embryo morphology by microspores in the culture media older than the late uninucleate cytological stage.

The embryogenic response of *Brassica* microspores to culturing has been observed as a dominant genetic trait (Chuong *et al.* 1988a). The inability of plants from the later backcross generations (i. e. BC_2F_1 and BC_2F_2) to produce large amounts of embryos upon culturing signaled the absence of the dominant embryogenic trait in the *B. juncea* and *B. rapa* parental lines used in the interspecific crossing scheme. An additional backcross generation between the BC_2F_2 plants and an embryogenic *B. rapa* line may have resulted in transfer of the dominant embryogenic trait into the *B. juncea/B. rapa* interspecific hybrid lines, resulting in higher embryo production frequencies upon culturing.

The studies of Chuong *et al.* (1988a) on embryo production in *B. napus* L. included the effects of genotype, inflorescence age, and bud developmental stage. Yield and frequency of normal embryo production were found to be genotype dependent for the

different varieties and individual plants cultured. Optimum age for bud sampling, for producing the highest frequency of embryos, was approximately one week after opening of the first flower on the main raceme. Dunwell and Cornish (1985) also obtained the highest embryo production after culturing of inflorescences of younger *B. napus* plants. Takahata *et al.* (1991) investigated the effects of donor plant age and inflorescence age with results differing from those of the previous studies in *B. napus* (Dunwell and Cornish 1985, Chuong *et al.* 1988a). Late uninucleate microspores from older plants (7-month-old) produced greater average embryo yields than microspores of the same cytological stage from younger plants (2 and 3 months old). The average efficiency of embryogenesis for plants of the three age groups, from oldest to youngest, were 5.67, 3.68, and 3.44%. The relative age of the inflorescences on the older and younger plants also affected the embryogenic response (Takahata *et al.* 1991). The highest embryo yields came from the younger racemes of 7-month-old plants and the older racemes from 2- and 3-month-old plants. The developmental stage of the microspores in new inflorescences of the younger plants was considered to be too early, while late uninucleate stage microspores were present in relatively high frequencies in the new inflorescences of 7-month-old plants and the old inflorescences of plants aged 2 and 3 months. Chuong *et al.* (1988a) found buds from both the main and lateral racemes produced similar embryo yields when the petal/anther ratio and similar bud size criteria were used to select for late uninucleate microspores.

Kott *et al.* (1988) studied the relationship between the cytological stage of microspores and their embryogenic response to *in vitro* culturing. From correlations of bud, anther, microspore, and nuclear size at each cytological stage of microspore

development, late uninucleate stage microspores were found to produce the highest number of cotyledenous embryos per sample of the two genotypes cultured (approximately 1300/anther). The relative bud size corresponding to the late uninucleate stage was observed to be genotype-specific between the two lines. In *Brassica* species, the optimal bud size can be estimated using the petal/anther ratio. Reliability of correlations between bud size, petal/anther ratio, and cytological stage for selection of late uninucleate microspores has been shown for several *Brassica* varieties and genotypes (Chuong *et al.* 1988a, Fan *et al.* 1988, Kott *et al.* 1988). Based upon cytological staging, microspore concentration adjustment, and reliability of bud size in estimating the late uninucleate stage, reduced torpedo-shaped embryo yields cannot be definitively attributed to inadequate bud selection criteria.

Kott and Polsoni (1988) noted reduced embryo yields and altered embryo morphology when liquid media from a culture consisting of binucleate stage microspores was used to inoculate highly embryogenic cultures of late uninucleate microspores. Changing the NLN media after culturing was shown to increase the frequency of normal embryo development. The MC protocol developed by Baillie *et al.* (1992) for *B. rapa* includes a media exchange step to be performed 24 hours after the culture event.

2.5 Conclusions

The induction of high frequencies of torpedo-shaped embryos (i. e. the type of embryo most likely to form plantlets) through *in vitro* culturing of microspores from the BC_1F_1, BC_1F_2, BC_2F_1, and BC_2F_1 *B. juncea/B. rapa* interspecific hybrid plants was not achieved in the current study. Haploid embryos that formed into plantlets were initially

composed of callus-like masses of undifferentiated cells. These cell masses required continuous subculturing on supplemented media to stimulate the initiation of a shoot-root axis and, eventually, the first true-leaf. The microspore culture technique was not only expected to balance genic and chromosomal abnormalities in *B. juncea/B. rapa* interspecific hybrids, but the initiation of homozygosity at all genetic loci would have ensured that the yellow seedcoat color and self-compatibility traits would not segregate in the DH progeny. Since there were only 4 DH_1 plants/lines formed, each with a small quantity of seed, the planned 2-year, two-location field trial could not proceed. Instead, seed from 21 yellow-seeded BC_2F_2 *B. juncea/B. rapa* interspecific hybrid lines was planted in single 6-meter observation rows during the 1999 field season. Seedcoat color, days-to-first flower, and days-to-maturity were recorded and open-pollinated seed was selected from individual plants for seed quality analyses (See Chapter 5).

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Chapter 3

Cytological examination and RAPD-DNA marker analysis of

BC$_2$F$_2$ *B. juncea/B. rapa* interspecific hybrid lines.

3.1 Introduction

Molecular marker technology can verify the presence of genetic material from parental species in *Brassica* interspecific hybrids, while cytological examination of pollen mother cells (i. e. PMCs) during meiosis can reveal the chromosomal state of individual interspecific hybrid plants.

Genome-specific and species-specific genomic differences at the deoxyribonucleic acid (DNA) level can be detected using one of several molecular marker technologies. The random amplification of polymorphic DNA (RAPD) technique uses arbitrary oligonucleotide sequences as primers for the polymerase chain reaction (PCR). Welsh and McClelland (1990) and Williams *et al.* (1990) simultaneously created the RAPD-PCR reaction to differentiate closely related genotypes, sometimes differing by only a single basepair or a short stretch of nucleotides. Binding of the arbitrary primers within 5 kilobasepairs, followed by elongation with DNA polymerase, separation of the hydrogen-bonded DNA strands by heat denaturation, and multiple repetitions of this process, the PCR cycle, results in the formation of large amounts of several different (sized) DNA fragments. When there is a difference in the nucleotide sequence to which the primer binds, the corresponding DNA entity will not be produced. Polyacrylamide gel electrophoresis is used to separate out the PCR products based on their relative size, which affects their ability to migrate through the electric field applied to the agarose-

solidified polyacrylamide separating gel. Using a fluorescent dye (such as ethidium bromide), the DNA can then be seen as a series of bands. The dominant nature of the primer activity (i. e. presence or absence of bands) limits the ability of the RAPD-PCR technique to differentiate between distinct alleles, but efficiently detects polymorphisms between genotypes, species, and/or genomes (Quiros *et al.* 1991, Thiagarajah *et al.* 1994, Struss *et al.* 1995).

RAPD markers have been used to verify the presence of genetic material from both parental species in *B. juncea/B. napus*-derived interspecific hybrids (Thiagarajah *et al.* 1994), to detect polymorphisms between *B. napus* and *B. nigra* (Struss *et al.* 1995), and to develop *B. nigra* (B-genome)-specific primers and locate their positions on specific chromosomes in *B. napus-B. nigra* addition lines (Quiros *et al.* 1991).

Brassica species represent an ascending order of aneuploids derived from a common progenitor, with haploid chromosome complements ranging from n=7 to n=13 (Manton 1932) and secondary balancing of polyploids (Catcheside 1934, Alam 1936, Haga 1938). The use of chromosome characters such as absolute length, symmetry of arms, and shape of the heterochromatic region provide evidence for a *Brassica* ancestral chromosome number of x=6 (Röbbelen 1960). Catcheside (1937), from studies on secondary bivalent pairing at meiosis in *B. oleracea*, theorized the six chromosome *Brassica* ancestor arose after the fusion of two of the chromosomes of a hypothetical x=7 primitive haploid ancestor of the *Cruciferae*. Cytological observations supporting the x=6 hypothesis were found in the meiotic behavior of the A- and C-genome chromosomes in *Brassica napocampestris* (2n=58, AAAACC), an auto-allohexaploid derived from the cross of *B. napus* and *B. rapa* (*syn. B. campestris*) (Nwantiki 1970). The arrangement and

resolution of the C-genome chromosomes during metaphase I in *B. napocampestris* was used to establish a basic chromosome complement of six for *B. oleracea*. An identical analysis of the backcross progeny of the F_1 *B. napocampestris* hybrids to *B. rapa* (*syn. B. campestris*)-types verified the x=6 ancestral state for the A-genome. The genomic events thought to have occurred in the evolutionary divergence of the three closely related *Brassica* elemental genomes were the spontaneous doubling of the six-chromosome prototype along with the loss of entire chromosomes, followed by the extensive duplication and rearrangement of the remaining chromosomes (Sikka 1940). The three diploid genomes contain variable copy numbers of the rearranged homologous chromosome segments originally present in the ancestral genome.

Chen and Heneen (1991) challenged the validity of the x=6 hypothesis, on purely theoretical grounds, suggesting an x=3 ancestral *Brassica* genome. Preliminary evidence for the 3-chromosome *Brassica* ancestor was based upon secondary meiotic bivalent associations in *B. oleracea* (Hussein and Abobakr 1976). Recently, the advent of molecular marker technology has allowed Lagercrantz and Lydiate (1996) to provide evidence of three complete, yet highly rearranged copies of a hexaploid ancestral genome in *B. nigra* (BB, 2n=16). The three diploid species are expected to contain different, highly reorganized versions of the hexaploid complement in its entirety. A combination of fission, fusion, and translocation events between entire chromosomes or chromosome segments of the hexaploid progenitor can account for the evolution of the A, B, and C *Brassica* elemental genomes (Lagercrantz and Lydiate 1996).

Small chromosomes make the determination of chromosome number in *Brassica* interspecific hybrids difficult. Therefore, the ability to stain the heterochromatic regions

without losing detail in the euchromatic regions is of great importance. Stringam (1970) added basic ferric acetate, a mordant, to Newcomer's fixative solution (Newcomer 1953) to improve the chromosome staining capabilities of acetocarmine.

Verifying the genic and chromosomal state of *Brassica* interspecific hybrid lines must be carried out to ensure that a newly introgressed trait has been incorporated into the genome of the reconstituted recipient species or interspecific hybrid line. Chromosome addition lines may appear to hold an introgressed trait, but these traits disappear with chromosome loss. Segregation and eventual loss of *B. juncea*-type complete resistance from *B. napus/B. juncea* interspecific hybrid chromosome addition lines (Roy 1984) illustrates the importance of cytological analysis following interspecific hybridization.

Pollen fertility estimates for interspecific hybrid lines indicate the percentage of PMCs likely to contain a balanced set of chromosomes. In the *Brassicaceae*, fertile pollen grains can be distinguished from immature and underdeveloped sterile pollen using the procedure developed by Lesley and Lesley (1939). In the presence of acetocarmine, the cytoplasm of fertile, well-formed pollen grains becomes red, while sterile microspores fail to absorb the stain, thereby remaining clear. As the frequency of meiotic non-disjunction increases in the presence of chromosomal and genic abnormalities, reduced proportions of viable pollen grains are expected in interspecific hybrid plants. Reduced pollen fertility has been correlated with the genetic disturbances (*e. g.* bridge-fragments, univalent chromosomes) commonly found in *Brassica* interspecific hybrids (Roy 1977, Prakash and Chopra 1990, Thiagarajah *et al.* 1994).

3.2 Materials and Methods

3.2.1 RAPD-PCR screening

Seed from the 21 BC_2F_2 *B. juncea/B. rapa* interspecific hybrid lines used to sow the 1999 field observation trial, *B. rapa* parental line 97-12929, and *B. juncea* parental lines 94-644 and 95-1600 were germinated on sterile silica sand (Sil Silica Inc. Edmonton-Calgary, Alberta – Grade Sil.1) in 60 x 15-mm Petri plates at room temperature on the bench surface. Four seedlings per line, or the maximum number available, were transferred to six-inch pots 3-5 days after plating. Plants were grown in soil-free media (Stringam 1971) in a Conviron growth cabinet (Model PGR15) under a 16 hour photoperiod (with a light intensity of 425-450 μmol m^{-2} s^{-1} of photosynthetically active radiation), with light/dark periods at 18 ± 2°C/ 15 ± 2°C, and were fertilized biweekly with 20-20-20 fertilizer. Leaves collected from plants at the 3-leaf stage were wrapped in aluminum foil and frozen in liquid nitrogen, lyophilized, and stored in a sealed plastic bag at -20°C until DNA extraction. After tissue harvest, plants were transferred to a greenhouse with day/night temperatures of approximately 20°C/ 15°C, a 16 hour photoperiod, and natural light supplemented with high intensity sodium vapor lights (HID Sylvania, Canada) producing a light intensity of 425-450 μmol m^{-2} s^{-1} of photosynthetically active radiation at the bench surface. Self-pollinated seed was obtained by enclosing plants within 28 x 55 cm plastic bags (Cryovac Canada, Inc.) for the entire flowering period. During this time plants were fertilized biweekly with 20-20-20 fertilizer. Leaf-tissue selected from only a single plant per line was crushed in liquid nitrogen using a chilled mortar and pestle. DNA extraction and quantification, the PCR-RAPD reaction, and agarose gel electrophoresis proceeded as described by Sommers

(1997). An exception was the preparation of a bulk primer reaction mixture, which was used to simultaneously screen all of the *B. juncea/B.* rapa interspecific hybrid and parental lines for polymorphisms on 44 lane, 1.4% (w/v) agarose gels. The random 10-basepair oligonucleotides used as arbitrary primers in the PCR-RAPD reaction were developed at the University of British Columbia (Table 3.1). A minimum of 2 reactions were performed for each primer. Amplified DNA was separated using a 75-120 volt current applied for periods up to 3 hours to ensure sufficient separation of amplified DNA fragments of different lengths. DNA bands were detected directly from the agarose gel by ethidium bromide (5 µl/100ml of agarose) fluorescence under UV light (254 nm). Gel images were captured on type 55 Polaroid film (Sambrook *et al.* 1989).

Table 3.1 University of British Columbia RAPD oligonucleotide primer identification numbers and nucleotide sequences (set #1).

Primer	Nucleotide sequence	Primer	Nucleotide sequence
81	5'-GAG CAC GGG G-3'	91	5'-GGG TGG TTG C-3'
82	5'-GGG CCC GAG G-3'	92	5'-CCT GGG CTT T-3'
83	5'-GGG CTC GTG G-3'	93	5'-GGG GGG AAA G-3'
84	5'-GGG CGC GAG T-3'	94	5'-GGG GGG AAC C-3'
85	5'-GTG CTC GTG C-3'	95	5'-GGG GGG TTG G-3'
86	5'-GGG GGG AAG G-3'	96	5'-GGC GGC ATG G-3'
87	5'-GGG GGG AAG C-3'	97	5'-ATC TGC GAG C-3'
88	5'-CGG GGG ATG G-3'	98	5'-ATC CTG CCA G-3'
89	5'-GGG GGC TTG G-3'	99	5'-ATC CCC TGG G-3'
90	5'-GGG GGT TAG G-3'	100	5'-ATC GGG TCC G-3'

3.2.2 Cytology

Individual plants from the 21 BC_2F_2 *B. juncea/B. rapa* interspecific hybrid lines used to sow the 1999 field observation trial, *B. rapa* parental line 97-12929, and *B. juncea* parental lines 94-644 and 95-1600 were grown in soil-free media (Stringam 1971) in a Conviron growth cabinet (Model PGR15) under a 16 hour photoperiod (with a light intensity of 425-450 µmol m^{-2} s^{-1} of photosynthetically active radiation), with light/dark periods at 18 ± 2°C/ 15 ± 2°C. Plants were fertilized biweekly with 20-20-20 fertilizer. Primary bud clusters were collected at approximately 10:00 am on the day when the primary bud cluster was just emerging from the boot. Buds were immediately immersed in Newcomer's solution (Newcomer 1953) containing basic ferric acetate, placed in the dark for 24 hours at room temperature, then stored at 4°C until analyzed. The basic ferric acetate, which serves as a mordant to improve staining in plants with small chromosomes (Stringam 1970), was prepared according to Kubik (1999) and added at a 3% (w/v) ratio to the Newcomer's solution.

Buds were removed from the fixative and entire clusters were placed in a glass petri dish. Periodic addition of 70% (v/v) ethanol prevented the buds from dessication during examination. Individual anthers were removed, placed on a glass microscope slide, and macerated with an iron needle in a single drop of 1% (w/v) acetocarmine. The bud size corresponding to diakinesis or early metaphase I in the PMCs was determined using a light microscope under 200X magnification. Tapetal debris was removed from slides containing PMCs at the correct cytological stage, a glass coverslip was placed over the acetocarmine droplet, and the underside of the slide was steamed. Heated slides were inverted and gently pressed onto a single layer of tissue paper to flatten the PMCs and fix them to the glass surface of the slide. Clear nail polish was used to temporarily seal the coverslip prior to observation under oil immersion at 600X or 1000X magnification. A minimum of seven PMCs were examined to determine the number of chromosomes present at diakinesis or early metaphase I of meiosis for each of the *B. juncea/B. rapa* interspecific hybrid lines and the *B. juncea* and *B. rapa* parental lines. PMCs with cytological stages ranging from anaphase I to anaphase II where checked for aberrant chromosome behaviors such as chromosome bridge-fragments, laggards, and non-disjunction. PMCs with distinct, non-overlapping chromosomes were photographed under oil immersion at 600X and/or 1000X magnification using a 35 mm camera (Olympus C-35AD) on an Olympus light microscope (Model BH-2).

3.2.3 Pollen fertility

Viable pollen production was determined for the 21 BC_2F_2 *B. juncea/B. rapa* interspecific hybrid lines used to sow the 1999 field observation trial, *B. rapa* parental line 97-12929, and *B. juncea* parental lines 94-644 and 95-1600. Four flowers were sampled from individual greenhouse-grown plants representing each line. Pollen from one anther per flower was brushed onto a glass microscope slide and immersed in 1 or 2 drops of 3% (w/v) acetocarmine. A glass coverslip was placed over the droplet prior to counting the number of well-formed viable pollen grains, which were stained red by the acetocarmine, and the sterile, unstained pollen grains. Counts were made under 200X magnification in four separate, non-overlapping fields of view. Results were reported as percentage viable pollen, which was calculated by dividing the number of viable pollen grains by the total number of pollen grains scored. Standard errors were also reported.

3.3 Results

3.3.1 RAPD-PCR screening

From the 20 University of British Columbia oligonucleotide primers used to screen the 21 BC_2F_2 *B. juncea/B. rapa* interspecific hybrid lines, 8 of the primers detected a total of 11 polymorphisms between the *B. juncea* and *B. rapa* parents. Eight of the 11 polymorphisms segregated as *B. rapa*-specific bands within the interspecific hybrid lines. These uninformative polymorphisms were produced by primers 84, 87, 88, 96, and 98, where a band/fragment polymorphic between *B. rapa* and *B. juncea* was not observed to segregate within the 21 BC_2F_2 *B. juncea/B. rapa* interspecific hybrid lines. Polymorphisms of this type did not reveal the presence of DNA from both of the parental

species in the interspecific hybrid lines. The 3 remaining polymorphisms were considered to be informative because the differences between the parents were also observed among the interspecific hybrid lines. Primer 85 produced a 1.8kb fragment associated with *B. juncea* in 3 of the 21 BC_2F_2 interspecific hybrid lines (data not shown). This 1.8kb band in lanes 2, 3, and 18 (lines 98-2205-3, 98-2205-4, and 98-2220-2) was absent from the remaining 18 BC_2F_2 interspecific hybrid lines and the *B. rapa* parent. Primer 96 produced a 625bp fragment that was only observed in the *B. juncea* parent and a single interspecific hybrid line (lane 1 – line 98-2205-1) (Fig. 3.1). Primer 97 produced a 1.2kb fragment in the *B. rapa* parent that was absent in both *B. juncea* parental lines and present in each of the 21 interspecific hybrid lines except for lanes 5 and 6 (lines 98-2209-1 and 98-2209-5) (data not shown).

Table 3.2 RAPD-PCR polymorphisms and fragment lengths (in basepairs or kilobasepairs) between *B. juncea* (94-644 and 95-1600) and *B. rapa* (97-12929) parental lines and 21 BC_2F_2 *B. juncea/B. rapa* interspecific hybrid lines.

Primer	Fragment Length	*B. rapa* parent	*B. juncea* parent	21 BC_2F_2 *B. juncea/B. rapa* interspecific hybrid lines
84	1.9kb fragment	Present	Absent	Present in all
85	1.8kb fragment	Absent	Present	Absent in all except lanes 2, 3 and 18
87	2.0kb fragment	Present	Absent	Present in all
88	1.5kb fragment	Present	Absent	Present in all
96	625bp fragment	Absent	Present	Absent in all except lane 1
96	600bp fragment	Present	Absent	Present in all
96	575bp fragment	Absent	Present	Absent in all
97	1.2kb fragment	Present	Absent	Present in all except lanes 5 and 6
98	525bp fragment	Absent	Present	Absent in all
98	550bp fragment	Present	Absent	Present in all
98	1.0kb fragment	Present	Absent	Present in all

Figure 3.1   RAPD-PCR screening results for polymorphisms between *B. juncea* (94-644 and 95-1600) and *B. rapa* (97-12929) parental lines, and segregation of *B. juncea*-associated fragments in 21 BC_2F_2 *B. juncea/B. rapa* interspecific hybrid lines. Lanes 1-21 (from left): BC_2F_2 *B. juncea/B. rapa* interspecific hybrid lines 98-2205-1, 98-2205-3, 98-2205-4, 98-2207-1, 98-2209-1, 98-2209-5, 98-2210-2, 98-2210-3, 98-2211-2, 98-2213-2, 98-2213-3, 98-2214-4, 98-2215-2, 98-2216-1, 98-2217-2, 98-2219-2, 98-2219-5, 98-2220-2, 98-2222-4, 98-2253-1, and 98-2253-5; lane 22: *B. rapa* parental line 97-12929; lane 23: *B. rapa* parental line 97-12929 (self-pollinated); lane 24: *B. juncea* parental line 95-1600 (self-pollinated); lane 25: *B. juncea* parental line 95-1600 (self-pollinated). Primer 96 produced a 625bp band (specified by arrow to right of gel) in *B. juncea* parental lines (lanes 24 and 25) and BC_2F_2 *B. juncea/B. rapa* interspecific hybrid line 98-2205-1 (lane 1). This fragment was absent from *B. rapa* parental lines (lanes 22 and 23) and the other 20 BC_2F_2 *B. juncea/B. rapa* interspecific hybrid lines.

Kbp

3.0
2.0
1.0
0.5

←

-------------------------*B. juncea/B. rapa* interspecifics------------------------- --*B. r*-- --*B. j*--

3.3.2 Cytology

Each of the 21 BC_2F_2 *B. juncea/B. rapa* interspecific hybrid lines, and *B. rapa* parental line 97-12929, contained 10 bivalent chromosome associations at diakinesis and early metaphase I of meiosis, while *B. juncea* parental lines 94-644 and 95-1600 regularly produced 18 bivalents during meiosis (Fig. 3.2, Table 3.3).

Chromosome stickiness was observed in *B. rapa* parental line 97-12929 and in 17 of 21 BC_2F_2 *B. juncea/B. rapa* interspecific hybrid lines (Fig. 3.2C, Table 3.3). Stickiness was also observed in *B. juncea* parental lines 94-644 and 95-1600.

Chromosome bridges were observed in 6 of 21 BC_2F_2 *B. juncea/B. rapa* interspecific hybrid lines (Fig. 3.3, Table 3.3), but not in the *B. rapa* or *B. juncea* parental lines. In lines 98-2209-5 and 98-2220-2, only 2 meiotic cells contained bridges out of several hundred anaphase I PMCs observed. The frequency of bridges from the other interspecific hybrid lines were 23, 25, 13, and 43% for lines 98-2205-3, 98-2210-3, 98-2217-2, and 98-2253-5, respectively. The number of PMCs observed at anaphase I for each of the preceding lines were 13, 20, 32, and 37, respectively.

Laggards were only noted in a single telophase II PMC from line 98-2210-2 (Table 3.3). This PMC contained four separate groups of 9 chromatids at the 4 poles of the cell after anaphase II. The remaining chromatids appeared to form a spatial relationship similar to the spacing between the four groups, but these 4 laggards were distinctly separate from the other 36 chromatids. As this laggard-type association was only observed once in the several hundred anaphase I and II PMCs sampled, this random event can be considered insignificant.

Table 3.3   Pollen fertility (in percent with standard error), chromosome stickiness, and the number of bivalent chromosome associations observed at diakinesis in PMCs sampled from the 21 BC_2F_2 B. juncea/B. rapa interspecific hybrid lines and the B. juncea (94-644 and 95-1600) and B. rapa (97-12929) parental lines.

Line	Species	# of PMCs	% with chromosome aberrations	# of bivalents	Stickiness	Pollen Fertility (Percent ± Error)	Observations
97-12929	B. rapa	10	n/a	10	yes	88 ± 1.4	Anaphase not observed
94-644	B. juncea	9	n/a	18	yes	97 ± 2.4	Anaphase not observed
95-1600	B. juncea	9	n/a	18	yes	98 ± 1.7	Anaphase not observed
2205-1	B. juncea/B. rapa	10	n/a	10	yes	87 ± 2.2	Anaphase not observed
2205-3	B. juncea/B. rapa	15	23.1	10	unknown	94 ± 0.8	Bridge-fragments
2205-4	B. juncea/B. rapa	14	n/a	10	yes	98 ± 1.7	Anaphase not observed
2207-1	B. juncea/B. rapa	15	n/a	10	unknown	87 ± 0.9	Anaphase I appeared normal
2209-1	B. juncea/B. rapa	7	n/a	10	yes	84 ± 3.3	Anaphase not observed
2209-5	B. juncea/B. rapa	7	<1.0	10	yes	93 ± 1.6	Bridge-fragments
2210-2	B. juncea/B. rapa	13	<1.0	10	unknown	81 ± 4.4	Anaphase I normal, laggard in Anaphase II PMC
2210-3	B. juncea/B. rapa	12	25	10	yes	76 ± 2.5	Bridge-fragments
2211-2	B. juncea/B. rapa	18	n/a	10	yes	97 ± 1.3	Anaphase not observed
2213-2	B. juncea/B. rapa	18	n/a	10	unknown	78 ± 7.4	Anaphase not observed
2213-3	B. juncea/B. rapa	15	n/a	10	yes	83 ± 1.9	Anaphase not observed
2214-4	B. juncea/B. rapa	13	n/a	10	yes	87 ± 0.8	Anaphase not observed
2215-2	B. juncea/B. rapa	8	n/a	10	yes	81 ± 2.9	Anaphase not observed
2216-1	B. juncea/B. rapa	16	n/a	10	yes	78 ± 3.1	Anaphase I appeared normal
2217-2	B. juncea/B. rapa	11	12.5	10	yes	86 ± 5.7	Bridge-fragments
2219-2	B. juncea/B. rapa	14	n/a	10	yes	83 ± 3.6	Anaphase not observed
2219-5	B. juncea/B. rapa	14	n/a	10	yes	83 ± 1.5	Anaphase not observed
2220-2	B. juncea/B. rapa	7	<1.0	10	yes	91 ± 3.8	Bridge-fragments
2222-4	B. juncea/B. rapa	9	n/a	10	yes	73 ± 4.1	Anaphase not observed
2253-1	B. juncea/B. rapa	9	n/a	10	yes	85 ± 2.8	Anaphase not observed
2253-5	B. juncea/B. rapa	7	43.2	10	yes	79 ± 2.4	Bridge-fragments

Figure 3.2 Bivalent chromosome associations observed during diakinesis in PMCs from *B. rapa* parent 97-12929, *B. juncea* parent 95-1600, and BC_2F_2 *B. juncea/B. rapa* interspecific hybrid line 98-2214-4. The *B. rapa* parental (3.2A) and BC_2F_2 *B. juncea/B. rapa* interspecific hybrid (3.2C) lines regularly formed 10 bivalents during diakinesis, while *B. juncea* parents (3.2B) always contained 18 bivalent chromosome associations. Arrow indicates chromosome stickiness.

A   4μ

B   4μ

C   4μ

Figure 3.3 Chromosome bridge association observed in an anaphase I PMC from BC$_2$F$_2$ *B. juncea/B. rapa* interspecific line 98-2253-5. Arrow indicates chromosome bridge association.

4μ

3.3.3 Pollen fertility

The pollen fertility estimate for the *B. rapa* parental line (97-12929) used as the backcross parent in the development of the 21 BC_2F_2 *B. juncea/B. rapa* interspecific hybrid lines was $88 \pm 1.4\%$ (Table 3.3). The *B. juncea* parents, 94-644 and 95-1600, had pollen viabilities of 97 ± 2.4 and $98\% \pm 1.7\%$, respectively. For the 21 BC_2F_2 *B. juncea/B. rapa* interspecific hybrid lines, 5 readings were higher than 90% with the highest being 98%, 11 lines had viability counts between 80 and 90 percent, and 5 lines displayed less than 80% fertility with 73% being the lowest value obtained. Standard error was reported for each viability estimate (by line).

3.4 Discussion

3.4.1 RAPD-PCR screening

Although evolutionarily divergent, the A-genomes of *B. juncea* (AABB, 2n=36) and *B. rapa* (AA, 2n=20) still contain chromosomes able to form into near-homologous pairs in F_1 *B. juncea/B. rapa* interspecific hybrid plants (Morinaga 1929b, Sikka 1940). Recombination between homeologous regions of paired chromosomes during meiosis I in interspecific hybrids allows (or accommodates) the introgression of genetic material between species.

After the first backcross, the BC_1F_1 interspecific hybrid plants would have contained at least 20 chromosomes (i. e. 10 *B. juncea/B. rapa* A-genome chromosome pairs) and a maximum of 28 chromosomes (i. e. 8 *B. juncea* B-genome univalents). With two backcross generations to the *B. rapa* parent, the *B. juncea* A-genome chromosomes could have been replaced by their *B. rapa* counterparts due to the preferential pairing of

homologous over non- or near-homologous chromosomes. If this were true, the probability of attaining recombined chromosomes in the BC_2F_2 *B. juncea/B. rapa* interspecific hybrid lines would be dependent upon the frequency of crossing-over between the *B. juncea* and *B. rapa* A-genome chromosomes in the F_1 interspecific hybrid, and the random pairing of chromosomes during the second backcross generation.

The RAPD-PCR reaction coupled with polyacrylamide gel electrophoresis allows closely related genotypes to be differentiated on a single basepair basis (Welsh and McClelland 1990, Williams *et al.* 1990). Genome-specific and species-specific differences in *Brassica* interspecific hybrid plants can be detected between the formative parents and within the interspecific progeny by RAPD-PCR analysis (Quiros *et al.* 1991, Thiagarajah *et al.* 1994, Struss *et al.* 1995). Since the RAPD-PCR technique functions on the ability of random primers to anneal to complementary DNA sequences, polymorphisms between genotypes, species, and/or genomes will be detected in a dominant manner (i. e. presence or absence of a band/fragment).

The random oligonucleotide primers used in the current study detected both informative and non-informative polymorphisms in the BC_2F_2 *B. juncea/B. rapa* interspecific hybrid lines. Differences between the *B. juncea* and *B. rapa* parental lines that did not segregate in the 21 BC_2F_2 *B. juncea/B. rapa* interspecific hybrid lines were considered non-informative due to their inability to detect *B. juncea* DNA introgressed into the *B. rapa* A-genome chromosomes. Three of 20 UBC oligonucleotide primers were able to detect genetic material from both parental lines in the 21 BC_2F_2 *B. juncea/B. rapa* interspecific hybrid lines (Fig. 3.1). In each of these cases the polymorphism between the *B. juncea* and *B. rapa* parental lines segregated within the *B. juncea/B. rapa* interspecific

hybrid lines. Detection of the *B. juncea*-associated fragment type (See Section 3.3.1 for explanation), and, therefore, *B. juncea* genetic material, in the *B. juncea/B. rapa* interspecific hybrid lines verified the interspecific hybridization event and the successful transfer of genetic material from *B. juncea* to *B. rapa*.

3.4.2 Cytology

In the cross of *B. juncea* (AABB, 2n=36) and *B. rapa* (AA, 2n=20) the F_1 interspecific hybrid is expected to contain 28 chromosomes (AAB, 2n=28) with ten bivalents (10_{II}) and eight univalents (8_I) on the metaphase plate during meiosis I. Morinaga (1929b) observed the $10_{II} + 8_I$ condition in each of the 40 PMCs from the cross *B. juncea* Coss. (*syn. B. cernua* Helmsl.) x *B. chinensis* L., a member of the n=10 *B. rapa* L. cytodeme (i. e. species containing common chromosomes in similar, yet evolutionarily distinct genomes (Harberd 1972)). Ramanujam and Srinivasachar (1943) performed reciprocal crosses of *B. juncea* and several 10-chromosome species of the *B. rapa* L. cytodeme and obtained hybrids with the same meiotic behavior. Sikka (1940) found the $10_{II} + 8_I$ condition in all but one of 80 cells examined. The exception was the presence of a quadrivalent ($1_{IV} + 9_{II} + 6_I$), signaling pairing between the homeologous regions of non-homologous chromosomes from the A- and B-genomes of *B. juncea* and the A-genome chromosomes of *B. rapa*. The common finding of ten bivalents and eight univalents in *B. juncea/B. rapa* PMCs supports the homologous relationship between the A-genome chromosomes of *B. juncea* and *B. rapa* (Morinaga 1929b, Sikka 1940, Ramanujam and Srinivasachar 1943).

In the formation of the BC_2F_3 plants observed in the 1999 field observation trial

two backcross and two self-pollination generations followed the initial cross of *B. juncea* x *B. rapa*. The F_1 interspecific hybrids should have contained (lines not checked), along with the 8 unpaired chromosomes of the *B. juncea* B-genome, 10 near homologous chromosome pairs composed of one entire set of both the *B. juncea* and *B. rapa* A-genomes. With each successive backcross to the *B. rapa* parent the proportion of plants containing two sets of the homologous chromosomes of the *B. rapa* A-genome were increased. Along with the self-pollination generations, the backcross steps were responsible for eliminating the univalent B-genome chromosomes present in the original interspecific hybrid plants. Random assortment of univalents during meiosis I and II and the preferential pairing of the homologous *B. rapa* A-genomes allowed the unpaired univalents to be eliminated and the 20-chromosome *B. rapa* A-genome diploid complement of the *B. rapa* parent to be reconstituted, respectively.

Instead of increasing the relative proportions of *B. rapa* genetic material in the *B. juncea/B. rapa* interspecific hybrids through conventional backcrossing, the B-genome may have been eliminated during meiosis. The selection of A-genome female gametes in the primary F_1 *B. juncea/B. rapa* interspecific hybrids, coupled with fertilization by the first backcross generation *B. rapa* parent, could have reestablished the diploid *B. rapa* genome in the BC_1F_1 generation, and, ultimately, in the BC_2F_2 interspecific hybrid lines. Kubik (1999) reported the selective production of 19-chromosome *B. napus*-type (AC) gametes in doubled haploid (DH) *B. napus/B. rapa* interspecific hybrid lines following a backcross to *B. rapa*. The preferential selection of the 19-chromosome *B. napus* gametes during microspore culture excluded the formation of 10-chromosome haploid embryos, which are the precursors for the 20-chromosome *B. rapa*-type doubled haploids. Song *et*

al. (1993) suggested that this form of genome stabilization, in doubled haploid plants, resulted in the formation of only full complements of haploid chromosome sets. Therefore, *B. napus/B. rapa* interspecific hybrids should produce both 10- and/or 19-chromosome gametes. Although the BC_2F_3 *B. juncea/B. rapa* interspecific hybrid lines studied were not derived from microspore culture, genomic stabilization of gametes in the interspecific hybrid plants may have influenced the selection of *B. rapa* gametes. In the 21 BC_2F_2 *B. juncea/B. rapa* interspecific hybrid lines (current study), the 18-chromosome *B. juncea*-type gametic complement was not observed. The production of 10-chromosome gametes indicates a species-specific or genotype-dependent influence may exist in gamete formation in *B. juncea/B. rapa* interspecific hybrids.

The *B. rapa* genomic complement was observed in PMCs of 21 BC_2F_2 *B. juncea/B. rapa* interspecific hybrid lines (Fig. 3.2C), and *B. rapa* parental line 97-12929 (Fig. 3.2A), as the regular formation of 10 bivalent chromosome associations during meiosis (Table 3.3). During diakinesis, the final stage of prophase before bivalents align on the metaphase plate, recombination structures (i. e. chiasmata) can be observed as physical associations between distal regions of daughter chromatids in homologous chromosome pairs (Fig. 2.3b). Crossing over between near homologous chromosomes of the *B. juncea* and *B. rapa* A-genomes in F_1 interspecific hybrid plants could result in trait introgression from *B. juncea* to *B. rapa*. A major practical implication of an ancestral progenitor for the diploid *Brassica* species is that the presence of homeologous (i. e. conserved) regions between the A-, B-, and C-genomes makes recombination between all of their chromosomes possible (Röbbelen 1960, Chen and Heneen 1991). Therefore, chromosome bridges observed in BC_2F_2 *B. juncea/B. rapa* interspecific lines could have

also resulted from B-genome chromosome introgression.

The chromosome bridges observed in the anaphase I PMCs of the BC_2F_2 *B. juncea/B. rapa* interspecific hybrid lines were formed when the cross-over junction between the two chromosomes failed to completely resolve and the chromosomes (still attached at the chiasma) began to migrate to opposite poles. Recombination between the homeologous regions of non-homologous chromosomes and the presence of chromosome inversions between homologous regions of identical paired chromosomes have been cited as the main cause of chromosome bridges and bridge-fragment associations. Olsson and Hagberg (1955) reported chromosome bridges and bridge-fragment associations in anaphase I and II *B. napus* PMCs and implicated inversions between homologous chromosomes, and the difficulties arising during their resolution, as the most likely cause for the observed chromosome abnormalities. Resolution of a paracentric bridge-fragment association during anaphase I or II would appear as a chromosome bridge stretching along the axis of chromosome division with the chromosome fragments being situated on the cell periphery, perpendicular to the migratory axis.

The physical abnormalities caused by chromosome bridges and bridge-fragment associations during meiosis I and II in *Brassica* interspecific hybrid lines regularly result in non-disjunction and gametic sterility (Roy 1977, Prakash and Chopra 1990, Thiagarajah *et al.* 1994). Pericentric inversions without resolution problems, that is, in the absence of chromosome bridges, can also lead to gametic sterility through chromosome duplications or deletions. As anaphase was only observed in 6 of 21 BC_2F_2 *B. juncea/B. rapa* interspecific hybrid lines, the frequency of chromosome bridges formed in the interspecific lines and the effect of inversions could not be explored

completely (Table 3.3).

Multivalent chromosome associations, i. e. tri-, quad-, or hexavalent, arise when crossing-over occurs between homeologous regions on non-homologous chromosomes. Kalasa-Balicka (1985) reported multivalent associations, as well as bridge-fragments and laggards, in *B. juncea/B. carinata* interspecific hybrids. In *Brassica* interspecific hybrids, multivalent structures are formed when highly duplicated or conserved regions on non-homologous chromosomes from different genomes recombine and become physically associated by chiasmata. Multivalent structures were not observed in any *B. juncea/B. rapa* interspecific hybrid lines or *B. juncea* and *B. rapa* parental lines. The absence of multivalents in BC_2F_2 *B. juncea/B. rapa* interspecific hybrid lines was expected as trivalent and quadrivalent associations have been rarely observed in PMCs of 28-chromosome *B. juncea/B. rapa* interspecific hybrids containing the *B. juncea* A- and B-genomes. Sikka (1940) found only a single quadrivalent association out of 80 PMCs observed in F_1 interspecific hybrids from the cross *B. juncea* x *B. rapa*.

Movement of unpaired univalent chromosomes from the metaphase plate to the poles of PMCs may lag behind the migration of recently separated chromosomes of bivalents. These laggards will appear between the 2 sets of dividing chromosomes during anaphase (I and/or II). None of these univalent-derived laggards were observed in PMCs of BC_2F_2 *B. juncea/B. rapa* interspecific hybrid lines because the 20 chromosomes present regularly formed ten bivalents during meiosis. However, in line 98-2210-2 a single anaphase II PMC apparently contained 4 laggards skewed away or partitioned from the other 36 chromatids, the latter in 4 distinct groupings of 9 chromatids (Table 3.3). Since this configuration was observed in only 1 PMC, it was likely a random event.

3.4.3 Pollen fertility

Pollen viability estimates can provide an indication of the chromosomal and genic state of *Brassica* interspecific hybrids (Roy 1977, Prakash and Chopra 1990, Thiagarajah *et al.* 1994). Of 21 BC_2F_2 *B. juncea/B. rapa* interspecific hybrid lines, 16 had pollen fertility estimates lower than the 88% value obtained for *B. rapa* parental line 97-12929. In the current study, the purpose of pollen fertility estimates was to ascertain whether this data could predict the presence and frequency of chromosomal abnormalities in the *B. juncea/B. rapa* interspecific hybrid lines. Because anaphase was not observed in all of the interspecific hybrid lines, the status and relative frequency of chromosome bridges and/or bridge-fragments and their relationship to pollen fertility, could not be determined definitively.

3.5 Conclusions

RAPD-DNA marker analysis of the 21 BC_2F_2 *B. juncea/B. rapa* interspecific hybrid lines verified the presence of species-specific DNA from both formative parental species, while cytological examination of PMCs sampled from these interspecific hybrid lines showed regular formation of 10 bivalent chromosome pairs during diakinesis. The chromosome bridges observed during anaphase I of meiosis in PMCs from 6 of 21 BC_2F_2 *B. juncea/B. rapa* interspecific hybrid lines, along with multivalent chromosome associations (none observed) and laggards (in a single anaphase II PMC), are usually indicative of meiotic abnormalities in *Brassica* interspecific hybrids (Kalasa-Balicka 1985). Since anaphase was not observed in all 21 BC_2F_2 *B. juncea/B. rapa* interspecific hybrid lines, chromosome bridges formed in the 6 hybrid lines could not be attributed to

inversions arising from genetic recombination between the near-homologous A-genome chromosomes of *B. juncea* and *B. rapa*. Although the status and relative frequency of chromosome bridge associations was unavailable for all of the BC_2F_2 *B. juncea/B. rapa* interspecific hybrid lines, the bridges observed in lines with pollen fertility estimates greater than 90% indicate that pollen fertility may be inadequate as a predictor of chromosomal abnormalities in *B. juncea/B. rapa* interspecific lines (Table 3.3).

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Disease screening of 21 BC₂F₃ *B. juncea/B. rapa* interspecific hybrid lines and their

formative parents for resistance to blackleg disease caused by *Leptosphaeria*

***maculans* and white rust disease caused by *Albugo candida*.**

4.1 Introduction

Two of the most important fungal diseases of commercial *Brassica* oilseed varieties in western Canada are blackleg disease of canola caused by *Leptosphaeria maculans* (Desmaz.) Ces. & de Not. (anamorph *Phoma lingam* (Tode:Fr) Desmaz.) and white rust disease of canola caused by *Albugo candida* (Pers. Ex Hook.) Kuntze.

L. maculans infection causes necrotic lesions on the leaves, pods, and stems of susceptible *Brassica* plants. Systemic spread of the pathogen can lead to formation of crown cankers near the base of the stem, blockage of water flow to leaves, premature ripening, and, sometimes, lodging (Rempel and Hall 1996). Genetic resistance is the most economically feasible strategy used to control blackleg in *Brassica* species (Rimmer and van den Berg 1992, Rempel and Hall 1996). Conventional breeding practices gave moderate levels of resistance with a trend to stronger resistance in *B. napus* varieties and winter types compared to *B. rapa* and spring type cultivars (Thurling and Venn 1977). Roy (1978) found *B. juncea* to be more resistant to blackleg disease than *B. carinata*, *B. napus*, and *B. rapa*. There are several *B. napus* varieties resistant to the prevalent blackleg isolates in western Canada, while only a few *B. rapa* varieties with high levels of resistance have been released. Microspore culture technique was used to produce the highly blackleg resistant *B. napus* cultivars Quantum (Stringam *et al.* 1995a) and Q2

(Stringam *et al.* 1999). The sources of the resistance gene(s) in Quantum (Stringam *et al.* 1995b) and Q2 (Stringam *et al.* 1999) were the Australian rapeseed cultivars Maluka and Shiralee, respectively. Rimmer *et al.* (1998) developed the *B. napus* variety Sentry, which is highly resistant to blackleg and adapted to the long- and mid-season canola growing zones of the Canadian Prairies, through a modified backcross strategy between the French variety Jet Neuf and a blackleg susceptible western Canadian *B. napus* canola quality cultivar.

Various races of *A. candida* infect members of the *Brassicaceae* family, including both vegetable and oilseed crops (Pound and Williams 1963). Although yield losses have been observed on susceptible *B. juncea* and *B. rapa* varieties (Petrie 1988), virulent forms of pathotypes causing white rust disease in Canadian *B. napus* cultivars are absent from North America (Fan *et al.* 1983), except for isolated cases of susceptibility to race 7v in cultivars originating from China (Downey and Rakow 1987). Visible symptoms of white rust disease of canola are expressed in two different forms. White pustules on stems, inflorescences, and, in heavy concentrations, on abaxial leaf surfaces; and staghead formation in terminal bud clusters. Stagheads, which form later in the growing season after systemic spread of the pathogen into the meristematic cells of the infected plant, result from uncontrolled cell division and cell growth (Fan *et al.* 1983).

In the *B. napus* cultivar Regent, three dominant genes at independent loci have conditioned white rust race 7 resistance since the mid-1930s (Fan *et al.* 1983). Although complete resistance to white rust race 2 can be provided by a single dominant gene in *B. juncea* (Ebrahimi *et al.* 1976, Tiwari *et al.* 1988) and *B. rapa* (Downey and Rimmer 1993), only moderate levels of resistance to race 7 are available in *B. rapa*. A high degree

of outcrossing may be responsible for the low-to-moderate level of white rust resistance in self-incompatible *B. rapa*.

Interspecific hybridization has been used to introgress disease resistance traits between important *Brassica* species. Gerdemann-Knörck *et al.* (1994) transferred blackleg resistance from *Brassica nigra* to *B. napus* through asymmetric somatic hybridization of protoplasts. Zhu *et al.* (1993) and Chevre *et al.* (1996) developed *B. napus-B. nigra* chromosome addition lines with complete resistance to blackleg disease in some reconstituted *B. napus* lines. Roy (1984) first reported transfer of B. *juncea*-type complete resistance into *B. napus*, but the interspecific hybrid-derived lines were actually *B. napus-B. juncea* addition lines still segregating for the *B. juncea* blackleg resistance trait. Crouch *et al.* (1994) successfully introgressed a monogenic form of blackleg resistance from a wild *B. rapa* selection into *B. napus* using a synthetic *B. napus* amphidiploid as an intermediate. Singh and Singh (1987) developed trigenomic *B. juncea/B. carinata* interspecific hybrids (ABBC, 4n=35) to facilitate the introgression of the white rust resistance trait of *B.* carinata into *B. juncea*. The successful trait transfer was probably accomplished through recombination between homologous B-genome chromosomes of the white rust resistance donor and recipient species.

The objectives of the current experiment were to determine blackleg and white rust disease responses in *B. juncea/B. rapa* interspecific lines and to study the introgression of high levels of blackleg resistance of *B. juncea* into *B. rapa*. Disease screening was performed on progeny of the 21 BC_2F_3 *B. juncea/B. rapa* interspecific hybrid lines grown in the field observation trial, *B. rapa* parental line 97-12929, and *B. juncea* parental lines 94-644 and 95-1600 (white rust screening only).

4.2 Materials and Methods

4.2.1 Introduction

Progeny from the 21 BC_2F_3 *B. juncea/B. rapa* interspecific hybrid lines derived from the field observation trial, and *B. rapa* parental line 97-12929, were screened for resistance to the prevalent *L. maculans* (Desmaz.) Ces. & De Not. and *A. candida* (Pers. Ex Hook.) Kuntze isolates present in western Canada. The *B. juncea* parental lines 94-644 and 95-1600 were only screened for white rust resistance because of limited seed availability. Cotyledonary assays developed by Bansal *et al.* (1994, 1999) were used to screen for blackleg and white rust resistance, respectively.

4.2.2 Blackleg disease screening

Seven days after sowing, seedlings were inoculated with a single pycnidiospore suspension prepared from 5 separate single-spored virulent *L. maculans* isolates (Bansal *et al.* 1994). Blackleg resistance screening proceeded using the cotyledon procedure developed by Bansal *et al.* (1994). Blackleg disease symptom ratings were performed by Mr. Vipan Bansal of the University of Alberta canola breeding program, using the disease severity scale (for cotyledons) described by Bansal *et al.* (1994) (Table 4.1). The disease severity index (DSI) was calculated from 2 separate repetitions of 8 plants for each line using the following formula: DSI = Σ (number of plants in disease severity category x disease severity category) / total number of plants inoculated. Standard error between the DSI ratings obtained from the 2 replicates was calculated.

Table 4.1 Blackleg disease severity categories and corresponding symptoms on cotyledons of seedlings inoculated with virulent *Leptosphaeria maculans* isolates (Modified from: Bansal *et al.* 1994).

Disease severity category	Disease symptoms
1	Necrotrophic hypersensitive response around the wound
2	Gray-green tissue collapse with distinct margin
3	Gray-green tissue collapse with diffused margin
4	Most of the tissue collapsed with pycnidia formation

4.2.3 White rust disease screening

Screening for resistance to races 2a and 7v of *A. candida* proceeded as described by Bansal *et al.* (1999), except for race 7v maintenance and increase on cotyledons of *B. rapa* cv. Torch. White rust disease responses on the cotyledons were observed 9 or 10 days after inoculation. Lines were considered resistant only if all of the cotyledons of the inoculated seedlings were free of pinhead-sized or coalescing pustules.

4.3. Results

All of the progeny of the 21 BC$_2$F$_3$ *B. juncea/B. rapa* interspecific hybrid lines derived from the field observation trial, and *B. rapa* parental line 97-12929, were resistant to the *L. maculans* isolates used to screen for blackleg disease (Table 4.2). The disease severity index (DSI) was reported as a single value for the two 8-plant replicates and ranged from 1.0 to 2.0 (where DSI >2.0 = susceptibility) (Table 4.1).

B. juncea parental lines 94-644 and 95-1600 were resistant to race 7v and susceptible to race 2a of *A. candida*, while *B. rapa* parental line 97-12929 and the 21 BC$_2$F$_3$ *B. juncea/B. rapa* interspecific hybrid lines were susceptible to race 7v and

segregated for resistant to race 2a (Table 4.2). Each of the BC_2F_3 *B. juncea/B. rapa* interspecific hybrid lines screened with the race 2a inoculum displayed both susceptible and resistant disease responses, except for lines 98-23705, 98-23707, and 98-23708 in which only susceptibility was observed (Table 4.2). The progeny of *B. rapa* parental line 97-12929 also segregated for resistance to race 2a with 10 susceptible and 8 resistant responses.

Table 4.2 Blackleg and white rust disease screening results for 21 *B. juncea/B. rapa* interspecific hybrid lines and *B. juncea* (94-644 and 95-1600) and *B. rapa* (97-12929) parental lines. a = *B. rapa* field check lines (98-23699, 98-23706, and 98-23713) represented by line 98-23699, b = bulked seed sample, c = two susceptible responses from 18 inoculations, Comm. Br. = Commercial Brown, DSI = disease severity index (where a value >2.0 represents susceptibility), n/a = not applicable, NS = not screened, R = resistant, S = susceptible, SE = standard error.

Line	Reference	Species	Blackleg		White rust race 2a		White rust race 7v	
			DSI	SE	Results	Rating	Results	Rating
Parent	94-644	*B. juncea*	NS	n/a	9S:0R	S	0S:9R	R
Parent	95-1600	*B. juncea*	NS	n/a	9S:0R	S	0S:9R	R
Parent[a]	97-12929	*B. rapa*	1.7	0.4	10S:8R	S	17S:0R	S
98-23695	98-2205-1	*B. juncea/B. rapa*	1.3	0.2	7S:5R	S	12S:0R	S
98-23696	98-2205-3	*B. juncea/B. rapa*	1.3	0.4	8S:10R	S	18S:0R	S
98-23697	98-2205-4	*B. juncea/B. rapa*	1.3	0.0	3S:14R	S	18S:0R	S
98-23698	98-2207-1	*B. juncea/B. rapa*	1.5	0.4	13S:4R	S	17S:0R	S
98-23700	98-2209-1	*B. juncea/B. rapa*	1.0	0.4	10S:2R	S	14S:0R	S
98-23701	98-2209-5	*B. juncea/B. rapa*	1.8	0.5	12S:3R	S	13S:0R	S
98-23702	98-2210-2	*B. juncea/B. rapa*	1.3	0.9	9S:3R	S	14S:0R	S
98-23703	98-2210-3	*B. juncea/B. rapa*	1.6	0.4	7S:9R	S	17S:0R	S
98-23704	98-2211-2	*B. juncea/B. rapa*	1.0	0.4	7S:5R	S	16S:0R	S
98-23705	98-2213-2	*B. juncea/B. rapa*	1.3	0.7	10S:0R	S	13S:0R	S
98-23707	98-2213-3	*B. juncea/B. rapa*	1.3	0.3	15S:0R	S	16S:0R	S
98-23708	98-2214-4	*B. juncea/B. rapa*	1.1	0.6	11S:0R	S	13S:0R	S
98-23709[b]	98-2215-2	*B. juncea/B. rapa*	1.6	0.6	11S:6R	S	17S:0R	S
98-23710	98-2216-1	*B. juncea/B. rapa*	1.4	0.5	10S:3R	S	16S:0R	S
98-23711	98-2217-2	*B. juncea/B. rapa*	1.0	0.2	14S:4R	S	16S:0R	S
98-23712	98-2219-2	*B. juncea/B. rapa*	1.7	0.8	13S:2R	S	17S:0R	S
98-23714	98-2219-5	*B. juncea/B. rapa*	1.3	0.4	12S:1R	S	14S:0R	S
98-23715	98-2220-2	*B. juncea/B. rapa*	2.0	0.8	12S:1R	S	18S:0R	S
98-23716	98-2222-4	*B. juncea/B. rapa*	1.8	0.0	10S:3R	S	15S:0R	S
98-23717	98-2253-1	*B. juncea/B. rapa*	1.4	0.3	14S:2R	S	17S:1R	S
98-23718	98-2253-5	*B. juncea/B. rapa*	1.1	0.3	11S:4R	S	17S:1R	S
White rust checks	Comm. Br.	*B. juncea*	NS	n/a	36S:0R	S	0S:24R	R
	Torch	*B. rapa*	NS	n/a	2S:16R	R[c]	48S:0R	S
	Cutlass	*B. juncea*	NS	n/a	30S:0R	S	NS	n/a
	Tobin	*B. rapa*	NS	n/a	NS	n/a	34S:2R	S
	Reward	*B. rapa*	NS	n/a	NS	n/a	18S:0R	S
Blackleg checks	Profit	*B. napus*	3.6	0.2	NS	n/a	NS	n/a
	Quantum	*B. napus*	1.3	0.6	NS	n/a	NS	n/a

4.4 Discussion

Susceptibility to race 7v of *A. candida* in the *B. juncea/B. rapa* interspecific hybrid lines could have resulted from extensive backcrossing to the susceptible *B. rapa* parent. After 3 crossing generations to *B. rapa*, i. e. the original hybridization event and 2 backcrosses, the interspecific hybrid lines would be theoretically composed of 87.5% *B. rapa* and 12.5% *B. juncea* DNA. Following the original interspecific cross, the proportion of *B. juncea* genetic material would decrease with successive backcross generations through random assortment and inheritance of chromatids and/or physical loss of univalents, during meiosis. The frequency of *B. juncea* A-genome chromosomes, homologous with the *B. rapa* A-genome chromosomes, would be reduced with each backcross generation to the *B. rapa* backcross parent, while the *B. juncea* B-genome chromosomes would be separated into only half of the gametes formed, because of a lack of a homologous pairing partner, and eventually be eliminated. The single gene dominant inheritance of resistance to race 7 in *B. juncea* (Ebrahimi *et al.* 1976, Tiwari *et al.* 1988) and *B. rapa* (Downey and Rimmer 1993) means that introgression of the genetic material for this trait from *B. juncea* to *B. rapa* would condition a resistant response in the corresponding interspecific hybrid line(s). Therefore, at least a few of the 21 BC_2F_3 *B. juncea/B. rapa* interspecific hybrid lines should have displayed resistant responses upon inoculation with race 7v, assuming that genetic recombination occurred between the *B. juncea* and *B. rapa* A-genome chromosomes in the F_1 interspecific hybrids. Excluding the two resistant ratings in lines 97-23717 and 97-23718 (Table 4.2), which apparently resulted from failure to place race 7v inoculum on the cotyledonary surface, the failure to obtain interspecific lines with a 3:1 ratio of resistant to susceptible responses indicates

that the gene conditioning resistance to race 7v in the *B. juncea* parental lines may be located on the *B. juncea* B-genome.

Segregation of resistance to race 2a of *A. candida* was observed in the BC_2F_3 *B. juncea/B. rapa* interspecific hybrid lines, the *B. rapa* parental line 97-12929, and the *B. rapa* check cultivar Torch (Table 4.2). Pound and Williams (1963) inoculated members of the *Brassicaceae* with physiological variants of *A. candida* in an attempt to develop a set of differential host species. The isolate causing white rust disease in *B. juncea* was designated race 2, while *B. rapa* served as differential host for race 7. Liu *et al.* (1996) referred to the genotypes of related *Brassica* species on which non-specific *A. candida* isolates were pathogenic as heterologous hosts. A mixture of resistant and susceptible responses upon inoculation of the *B. rapa* parent with the race 2a isolate indicated that some degree of cross-compatibility existed between this distinct *A. candida* isolate (i. e. race 2a) and the non-specific, non-differential *Brassica* host species (i. e. *B. rapa*). Three of the 21 BC_2F_3 *B. juncea/B. rapa* interspecific hybrid lines were found to be completely susceptible to race 2a based on the cotyledonary assay (Table 4.2). Sample sizes of 10, 15, and 11 cotyledonary-staged plantlets for lines 98-23705, 98-23707, and 98-23708, respectively, may not have been large enough to adequately express the genetic variation present at the race 2 locus/loci. Since lines 98-23705 and 98-23707 were both derived from BC_2F_2 line 98-2213, the lack of a resistant response in the 26 plantlets screened may indicate that these lines are no longer segregating for race 2a resistance. Assuming the race 2 resistance locus (or loci) have ceased to segregate, all progeny resulting from self-pollination of individual plants from these BC_2F_3 lines will be susceptible to race 2a isolates. Introgression of *B. juncea* genetic material coding for

susceptibility to race 2a into F_1 *B. juncea/ B. rapa* interspecific hybrid lines could account for the segregation of white rust race 2a resistance in these lines. However, since *B. rapa* parental line 97-12929 was not completely resistant when inoculated, and as selections were not made prior to the BC_2F_3 generation, introgression of race 2a resistance from *B. juncea* into *B. juncea/B. rapa* interspecific hybrid lines cannot be confirmed.

Blackleg disease severity indices (DSI) obtained for the 21 BC_2F_3 *B. juncea/B. rapa* interspecific hybrid lines and *B. rapa* parental line 97-12929 indicate the presence of moderate to high levels of blackleg resistance. Of the 2 *B. rapa* parents used to develop the BC_2F_1 generation, 97-12917 was not screened for blackleg resistance in the current study, but 97-12929 had a DSI of 1.7 (±0.4). These F_8 *B. rapa* selections were derived from an interspecific cross between *B. rapa cv.* Eclipse and an University of Alberta canola breeding line with *B. napus cv.* Tower in its pedigree. Kubik (1999) screened lines 97-12917 [95-442-3] and 97-12929 [95-439-2] for blackleg disease, using the same procedure developed by Bansal *et al.* (1994) and an inoculation mixture composed of the same single-spored virulent *Leptosphaeria maculans* isolates used to screen for blackleg resistance in the current study. The DSI values observed for 97-12917 and 97-12929 were 1.3 (±0.3) and 1.9 (±0.1), respectively. The DSI values for the blackleg check varieties from the current study and the blackleg greenhouse screening trial of Kubik (1999), in that order, were 1.3 (±0.6) and 1.0 (±0) for Quantum, and 3.6 (±0.2) and 3.6 (±0.1) for Profit. The moderate levels of blackleg resistance in the *B. rapa* parental lines 97-12917 and 97-12929 probably resulted from resistance retained from the *B. napus* cultivar, Tower.

It is unclear whether the high levels of adult blackleg resistance in *B. juncea* were introgressed into the *B. juncea/B. rapa* interspecific hybrids through homologous or homeologous recombination. The DSI values for the *B. rapa* parents were 1.3 ($\pm$0.3) for line 97-12917 [95-442-3] and 1.9 ($\pm$0.1) and 1.7 ($\pm$0.4) for line 97-12929 [95-439-2] (with the first 2 values reported by Kubik (1999) and the 3rd from the current study). The range of values may indicate or suggest that the DSI values observed for the *B. juncea/B. rapa* interspecific hybrid lines (ranging from 1.0 to 2.0) may have arisen through segregation at *B. rapa* loci alone, otherwise a more definite pattern of resistance versus susceptibility should have been observed. As the cotyledonary screening procedure of Bansal *et al.* (1994) is known to reliably correlate blackleg disease responses between seedling and adult stages, the moderate to high levels of resistant responses to *L. maculans* in the 21 BC_2F_3 *B. juncea/B. rapa* interspecific hybrid lines should also be observed in the field.

4.5 Conclusion

When screened for blackleg and white rust resistance at the cotyledonary stage, the 21 BC_2F_3 *B. juncea/B. rapa* interspecific hybrid lines contained moderate to high levels of resistance to *L. maculans*, were susceptible to race 2a of *A. candida*, and segregated for resistance to race 7v of *A. candida*, respectively. However, the parental contribution at the disease resistance loci (in terms of trait introgression) were not determined. The *B. rapa* parent, 97-12929, and each of the *B. juncea/B. rapa* interspecific hybrid lines had DSI values less than or equal to 2.0, indicating moderate to high levels of blackleg resistance. The *B. juncea* parents, 94-644 and 95-1600, were resistant to race

7v of *A. candida* and susceptible to race 2a of *A. candida*, as expected. *B. rapa* parent, 97-12929, and the 21 BC$_2$F$_3$ *B. juncea/B. rapa* interspecific hybrid lines were all rated as completely susceptible to race 7v, but when inoculated with race 2a these lines segregated for resistance. The ability of race 2a to infect the *B. rapa* parent 97-12929, a heterologous host, obscured the source of the partial white rust race 2a resistance within the *B. juncea/B. rapa* interspecific hybrid lines.

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Chapter 5

Introgression of agronomic, quality, and self-compatibility traits from *Brassica juncea* to *Brassica rapa* using *B. juncea/B. rapa* interspecific hybrid lines.

5.1 Introduction

Brassica juncea and *Brassica rapa* exhibit species-specific agronomic and quality traits and differ in expression of the self-incompatibility trait. Compared to *Brassica napus* and *B. rapa*, *B. juncea* has superior drought, heat and pod shattering resistance, contains a higher degree of resistance to blackleg disease, and the expression of yellow-seeded types is stable. *B. rapa* cultivars containing low to moderate levels of blackleg disease resistance and yellow-brown seed may be improved with introgression of the high levels of blackleg resistance and the yellow seedcoat color trait from *B. juncea*. Genetic recombination between the near homologous A-genome chromosomes of *B. juncea* (AABB, 2n=36) and *B. rapa* (AA, 2n=20) can serve as a bridge for trait introgression through interspecific hybridization (See Fig 1.1 and Section 1.2).

The standards for canola quality oilseeds are based on the amounts of erucic acid and glucosinolates, which are a long-chain fatty acid present in the oil and an antinutritional factor found in the meal fraction, respectively. *Brassica* cultivars currently grown must contain less than 2% erucic acid in the seed oil and no more than 30 micromoles per gram of aliphatic glucosinolates in the oil-free meal (Seeds Regulations of the Canadian Seeds Act 1989). Based on the guidelines of the Western Canadian Canola/Rapeseed Recommending Committee (WCC/RRC), cultivars currently being field tested in western Canada can contain no more than 0.5% erucic acid in the air-dried

oil-free meal fraction and the seed must either contain no more than 12 micromoles of total glucosinolates per gram of whole seed at 8.5% moisture content or contain total glucosinolate levels no greater than those of the designated check, whichever is higher (WCC/RRC Procedures and Appendices 1997). Commercial *B. rapa* canola quality cultivars have been available since 1977 when Candle was released for production in western Canada (Thomas 1984). The development of canola quality *B. juncea* for the southern prairies (Woods *et al.* 1991) will allow an additional 4-6 million acres to become suitable for canola production (Stringam personal communication).

The fatty acid profile in the commercial canola quality *Brassica* species, i. e. *B. napus* and *B. rapa*, are more nutritionally balanced than *B. juncea* in respect to the relative amounts of their major saturated (palmitic (C16:0) and stearic (C18:0)), monounsaturated (oleic (C18:1)), and polyunsaturated (linoleic (C18:2) and linoleic (C18:3)) fatty acids. A reduction in the ratio of saturated to unsaturated fatty acids and the amount of linolenic acid, and an increase in the proportion of linoleic to linolenic acid and the level of monounsaturated fatty acids, would further improve the nutritive and cooking qualities of the edible oil.

In addition to the fatty acid profile, the absolute amount of oil and the relative proportions of oil and protein present in the seed determine the oil quality of edible *Brassica* oilseed crops. Downey and Rimmer (1993) reported a range for the seed oil content of the *Brassica* oilseed species of 35-44% (on a dry weight basis) and Röbbelen and Thies (1980b) estimated the protein content of the dried oil-free meal at 40%. A negative correlation between oil and protein content heritability in *B. napus* (Grami *et al.* 1977) indicates that when attempting to improve the amounts of both components

simultaneously, selections should be made for the sum of oil and protein content. Based on literature and production statistics, Downey and Rimmer (1993) suggested that there is no upper limit in breeding for increased oil and protein content.

The content of oil and protein in the seed can also be increased through breeding for increased seed size and lower fibre and hull proportions. Hutcheson (1984) found fibre content to decrease proportionately when selection for larger seeds resulted in increased oil and protein content. The thinner hull and lower fibre content of yellow seeds, compared to brown and black, resulted in similar increases (Stringam *et al.* 1974). In this case, the thinner yellow seedcoat, and not the larger seed, was responsible for increasing the relative amount of oil and protein in the nutrient-rich embryo.

The well-balanced amino acid profile of the meal, composed of approximately 40% protein on a dry weight basis, is ideally suited as a feed supplement for beef, poultry, and swine (Röbbelen and Thies 1980b). Parameters for meal quality are the inclusion of no more than 30 micromoles of aliphatic glucosinolates per gram of air-dried oil-free meal, the relative amounts of the 20 essential, dietary amino acids, and the percentages of fibre and hull material present in the seedcoat. The breakdown products of glucosinolates, following enzymatic hydrolysis with myrosinase (i. e. thioglucoside glucohydrolase, E.C. 3.2.3.1), have been shown to cause mild to severe growth impairments and thyroid problems, leading to goiter, in poultry (Clandinin 1965) and swine (Bowland 1965). High glucosinolate rapeseed has been linked to reduced palatability of the meal by livestock (Bell and Devlin 1972), because the cleavage products of the sulfur-rich glucosinolates create a pungent, mustard-like odor and taste undesirable to beef and sheep.

The number of frost-free days required to reach physiological maturity is an important consideration when choosing a species for a particular planting region. On average, *B. rapa* reaches maturity about 10 days earlier than both *B. juncea* and *B. napus* (Stringam personal communication). The earlier maturity and higher level of shattering resistance of *B. rapa*, when compared to the higher-yielding *B. napus*, makes *B. rapa* better suited to the northern regions of the Canadian Prairies (Thomas 1984). The 'extra' days-to-maturity required for *B. napus* varieties to mature in the central and southern canola producing areas account for the average yield differential between *B. napus* and *B. rapa*.

In the *Brassicaceae*, seed color is controlled by a minimum of 2 genes (Stringam 1980, Chen and Heneen 1992, Rashid *et al.* 1994, Sing and Aruna 1994) with *B. napus*, *B. rapa*, and *B. juncea* predominately expressing seedcoat colors of black, yellow-brown, and brown or yellow-brown, respectively. Yellow seed color occurs naturally in varieties of *B. juncea* and *B. rapa*, but is absent in *B. napus* (Rashid *et al.* 1994). The reduced fibre content and hull thickness of yellow seed, when compared to brown- or black-seeded varieties, results in an increase in the relative amount of oil present and an increase in the meal protein content (Stringam *et al.* 1974).

The *Brassica* self-incompatibility system is under homomorphic monofactorial polyallelic control with sporophytic determination of pollen and pistil phenotypes. The *S*-locus, a single gene locus with multiple alleles, controls the *Brassica* self-incompatibility trait. Cellular interaction of *S*-locus specific glycoproteins (*S*LSGs) present in outer cell layers of the pollen and stigma leads to inability of self-pollen to germinate on the stigma surface (Nasrallah and Nasrallah 1988). In cases where

germination does occur, inhibition of pollen tube growth through the pistil inhibits fertilization of the ovules by pollen from the same plant.

The amphidiploid *Brassica* species (*B. carinata, B. juncea,* and *B. napus*) are self-compatible and reproduce (primarily) through self-pollination. The diploid species (*B. rapa, B. oleracea,* and *B. nigra*) are self-incompatible, suffer from inbreeding depression, and only produce small quantities of seed following self-pollination. As a result, *B. rapa* is reliant on breeding strategies that exploit the natural heterozygosity, and potential heterosis, present within this obligate outcrossing species.

The objective of the current experiment was to ascertain the utility of *B. juncea/B. rapa* interspecific hybrids for introgression of traits between these species. Selections were made for *B. rapa*-type lines (based on phenotypic characteristics and chromosome number) containing *B. juncea* traits. Research goals included improved blackleg disease resistance in *B. rapa* (See Chapter 4), the addition of 3-4 days to the days-to-maturity index of *B. rapa*, increased oil and protein content, decreased glucosinolate and erucic acid levels, improved nutritional quality of the *B. rapa* fatty acid profile (as outlined earlier in this section), and introgression of the non-segregating *B. juncea* yellow seedcoat color trait into *B. rapa*. Development of self-compatible *B. rapa* biotypes, able to avoid inbreeding depression, would allow for breeding techniques of self-pollinated crops to be implemented in *B. rapa* breeding programs.

5.2 Materials and Methods

5.2.1 Field observation trial

Seed from 21 BC_2F_3 *B. juncea/B. rapa* interspecific hybrid lines and *B. rapa*

parental line 97-12929 were sown at the University of Alberta Michener research farm on May 8, 1999 in single 6-meter rows spaced 28 inches apart. Primary bud clusters were selected for cytological analysis from 8 plants per line. Samples were collected at approximately 10:00 am on the day prior to emergence of the bud cluster from the shoot, immersed in Newcomer's solution (Newcomer 1953) containing 3% (w/v) basic ferric acetate, placed in the dark for 24 hours at room temperature, then stored at 4°C until analyzed. Single racemes of 8 randomly selected plants per line were bagged with 28 x 55 cm plastic bags (Cryovac Canada, Inc.) for the entire flowering period to produce self-pollinated seed. Self- and open-pollinated seed was collected from each selected plant for the determination of oil, protein, and glucosinolate content, fatty acid profile, and seedcoat color of the interspecific hybrid lines and the *B. rapa* parental line. Days-to-first flower and days-to-maturity observations were recorded.

5.2.2 Determination of oil, protein, and glucosinolate content

Oil, protein, glucosinolate, and seed moisture content were determined simultaneously by near infrared spectroscopy (NIR), using a Foss NIRSystem (Foss NIRSystems Ltd.). Four to 5.0 g seed samples were analyzed from two or three individual plants per BC_2F_3 *B. juncea/B. rapa* interspecific hybrid line and three selections from the *B. rapa* parental line, 97-12929, grown in the 1999 field observation trial. For *B. juncea* parental line 94-644, seeds from a single plant were subsampled three times because of limited seed quantities. For *B. juncea* parental line 95-1600 an inadequate amount of seed was available to test for oil, protein, and glucosinolate content. A quantity of seed less than 4.0 g provides insufficient coverage of the sample

test cell and results in a faulty scan because of the inability of the NIR system to differentiate between sample-filled and blank areas.

Following NIR analysis, content of oil, protein, glucosinolate, and seed moisture was calculated using the WINISI program (ISI – Infrasoft International LLC.). Oil content was reported as a percentage, on a whole seed, dry basis. Protein content was reported as a percentage, based on its proportion of the air-dried oil-free meal. Total glucosinolate content (i. e. all types with derivatives) was reported in micromoles per gram, as present in the oil-free meal, corrected to 8.5% moisture. Total glucosinolate content, on an oil-free meal basis, was determined by dividing one hundred times the micromoles per gram value for total glucosinolate content, on a whole seed, dry basis, by 100 minus the percentage oil content. Correction to 8.5% moisture was made by multiplying the total glucosinolates per gram (in micromoles), on an oil-free meal basis, by 0.92 (i. e. 100/108.5).

5.2.3 Fatty acid profile analysis

Relative fatty acid proportions were determined using the ISO 5508 (1990) procedure for animal and vegetable oils, following analysis of the hexane-extracted seed oil, with a Gas Liquid Chromatograph (HP model 5890 series 1), equipped with an autosampler. Proportions of the individual fatty acids present in the seed oil were determined for the same field-derived BC_2F_3 *B. juncea/B. rapa* interspecific hybrid and *B. rapa* plants used to determine oil, protein, and total glucosinolate content. Fatty acid profiles were also generated for *B. juncea* parental lines 94-644 and 95-1600. Individual fatty acid proportions were reported as relative percentages of the total fatty acid profile.

5.2.4 Days-to-first flower and days-to-maturity

The number of days required to reach physiological maturity and the days to first flowering were determined for the BC_2F_3 *B. juncea/ B. rapa* interspecific hybrid lines and the *B. rapa* parental line, 97-12929, grown in the 1999 field observation trial. Lines were considered to have reached first flower when approximately 10% of the plants displayed opened/flowering buds. Individual plants were physiologically mature when 30% of their seeds on the main raceme had undergone a seedcoat color change from green to brown, yellow, or yellow-brown. Entire lines were considered to be mature when 70% of their plants had undergone the requisite color change. Days-to-first flower and days-to-maturity values were reported in days, with the sowing date being set as day 0 (zero).

5.2.5 Seedcoat color

Seedcoat color was determined upon visible inspection of seed from each of the 21 BC_2F_3 *B. juncea/B. rapa* interspecific lines and the *B. rapa* parental line, 97-12929, grown in the 1999 field trial, and the *B. juncea* parental lines, 94-644 and 95-1600.

5.2.6 Self-incompatibility

The genetic state of the self-incompatibility trait (i. e. self-compatible or self-incompatible) in the 21 BC_2F_3 *B. juncea/ B. rapa* interspecific hybrid lines was determined during the 1999 field observation trial. Single racemes of 8 randomly selected plants per line were bagged with 28 x 55 cm plastic bags (Cryovac Canada, Inc.) for the entire flowering period to produce self-pollinated seed. Plant vigor and seed-setting behavior were observed in individual BC_1F_1, BC_1F_2, BC_2F_1, BC_2F_2, and BC_2F_3 plants.

5.3 Results

Values obtained for oil, protein, and glucosinolate content (Table 5.1), and the relative proportions of the nutritionally important fatty acids (Table 5.2) for individual plants from the 21 BC_2F_3 *B. juncea/B. rapa* interspecific hybrid lines were reported as an average value for each line. Results for these parameters were also reported for *B. rapa* parental line 97-12929 and *B. juncea* parental line 94-644.

The 21 BC_2F_3 *B. juncea/B. rapa* interspecific hybrid lines had an average oil content of 42.20% (range: 36-47%) and contained approximately 49.15% protein (range: 46.18-51.67%) and 22.84 ± 9.28 micromoles per gram (average) of total glucosinolates in the air-dried oil-free meal. The *B. rapa* parental line 97-12929 contained approximately 42.17% oil on a whole seed, dry basis, with a protein content estimate of 48.37% and an average total glucosinolate content of 20.91 micromoles per gram. Oil, protein, and glucosinolate content for *B. juncea* parental line 95-1600 were not determined as an insufficient amount of seed was available for this line, but breeding line 94-644 contained average values for these parameters of 35.09%, 46.06%, and 99.41%, respectively. Oil, protein, and glucosinolate content in the 21 BC_2F_3 *B. juncea/B. rapa* interspecific hybrid lines and *B. rapa* parental line 97-12929 were almost identical for each parameter. The values obtained for *B. juncea* parental line 94-644 were similar to those of the interspecific hybrid lines for protein content, but dissimilar for oil and glucosinolate content. The reconstitution of the 10-chromosome *B. rapa* genomic complement in the *B. juncea/B. rapa* interspecific lines through backcrossing, or genomic stabilization as proposed by Song *et al.* (1993), could provide a physical explanation for the similarities and contrasts discussed above (See Chapter 3).

The average oleic, linoleic, linolenic, erucic, and saturated fatty acid contents in the 21 BC_2F_3 *B. juncea/B. rapa* interspecific hybrid lines were 55.10, 24.91, 11.97, 0.06, and 6.04%, respectively, while the values for these nutritionally important fatty acids in *B. rapa* parental line 97-12929 were 54.33, 25.90, 11.73, 0.05, and 6.11%. The *B. juncea* breeding line, 94-644, contained mean oleic, linoleic, linolenic, erucic, and saturated fatty acid contents of 9.93, 18.81, 11.66, 44.68, and 4.98%, respectively, while *B. juncea* parental line 95-1600 displayed fatty acid readings of 26.49% oleic, 29.17% linoleic, 9.83% linolenic, 16.02% erucic, and 7.77% saturates. The nearly identical fatty acid values between the *B. juncea/B. rapa* interspecific lines and *B. rapa* parental line 97-12929 could have resulted from the reconstitution of the *B. rapa* genomic complement in the *B. juncea/B. rapa* interspecific lines (See Chapter 3). When compared with the fatty acid profiles of the 21 BC_2F_3 *B. juncea/B. rapa* interspecific hybrid lines (mean values) and *B. rapa* parental line 97-12929, the lower oleic acid and higher erucic acid contents of *B. juncea* parental lines 94-644 and 95-1600 could also be explained by reconstitution of the *B. rapa* genomic complement.

Results for seedcoat color, days-to-first flower, and days-to-maturity were reported for the 21 BC_2F_3 *B. juncea/B. rapa* interspecific hybrid lines and the *B. rapa* parental line, 97-12929, grown in the field observation trial (Table 5.3). The *B. juncea* parental lines, 94-644 and 95-1600, were not included in the 1999 field observation trial because of insufficient seed. Therefore, the days-to-first flower and days-to-maturity parameters could not be measured for lines 94-644 and 95-1600.

B. rapa parental line 97-12929 reached first-flower at approximately 45.67 days and physiological maturity after about 100 days (Table 5.3). The average days required to

reach first flower for the 21 BC_2F_3 *B. juncea/B. rapa* interspecific hybrid lines was approximately 47 ± 2 days, with a days-to-maturity average of 101 days (range: 95-107 days) (Table 5.3). Although the days-to-first flower and days-to-maturity mean values obtained for the *B. rapa* parental and *B. juncea/B. rapa* interspecific hybrid line were very similar, individual values at the lower and upper limits of the two parameter ranges could be used to select for reduced days-to-first flower and increased days-to-maturity, respectively. Seedcoat color in *B. juncea* parental lines 94-644 and 95-1600 was true-breeding for brown, while all progeny from the 21 BC_2F_2 and BC_2F_3 *B. juncea/B. rapa* interspecific hybrid lines developed yellow seeds.

Based on seed-setting behavior and vigor observations (data not shown), the 21 BC_2F_3 *B. juncea/B. rapa* interspecific hybrid lines did not display the symptoms of inbreeding depression, i. e. a loss in vigor and reduced pollen and ovule fertility. There was regular development of well-formed, seed-filled pods following self-pollination instead of the aborted ovules and stunted, seedless pods indicative of a self-incompatible response.

Table 5.1 Oil, protein, and glucosinolate content (in percent) reported as average values for 21 BC_2F_3 *B. juncea/B. rapa* interspecific hybrid lines and *B. juncea* (94-644) and *B. rapa* (97-12929) parental lines. a = field checks (98-23699, 98-23706, and 98-23713), b = bulk seed, c = statistical parameter calculated for 21 BC_2F_3 *B. juncea/B. rapa* interspecific hybrid lines. SE = standard error.

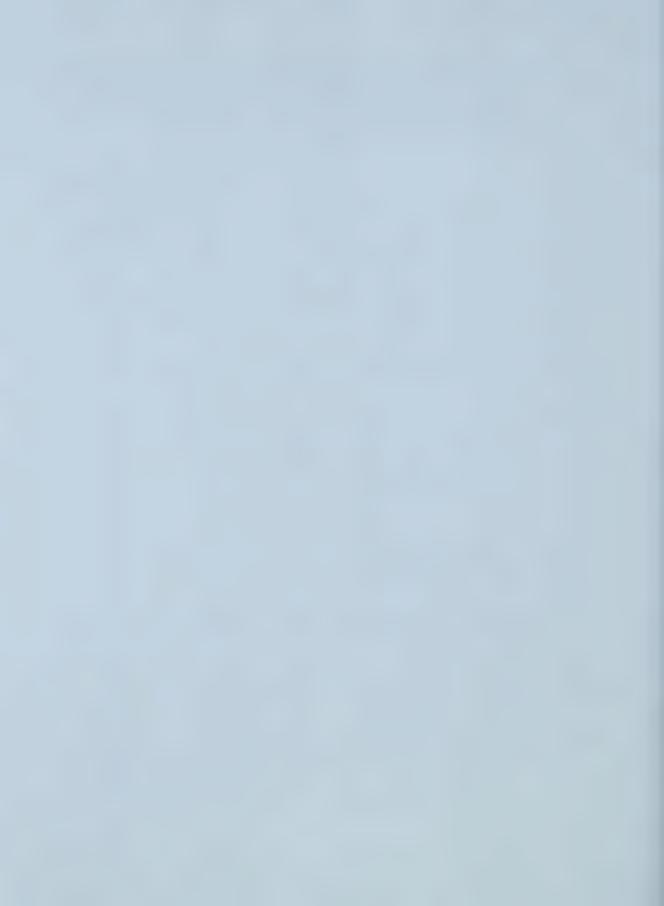| Field Line	Reference	Species	OIL Whole seed, dry basis (%)	PROTEIN Oil-free meal basis (%)	TOTAL GLUCOSINOLATES Oil-free meal basis, 8.5% moisture (micromoles/g)
Parent[a]	97-12929	*B. rapa*	42.17	48.37	20.91
Parent	94-644	*B. juncea*	35.09	46.06	99.41
98-23695	98-2205-1	*B. juncea/B. rapa*	39.04	48.64	36.97
98-23696	98-2205-3	*B. juncea/B. rapa*	44.36	46.30	25.22
98-23697	98-2205-4	*B. juncea/B. rapa*	42.02	49.48	22.28
98-23698	98-2207-1	*B. juncea/B. rapa*	40.61	49.01	30.25
98-23700	98-2209-1	*B. juncea/B. rapa*	43.35	51.67	13.21
98-23701	98-2209-5	*B. juncea/B. rapa*	43.17	49.82	20.30
98-23702	98-2210-2	*B. juncea/B. rapa*	42.21	48.72	18.61
98-23703	98-2210-3	*B. juncea/B. rapa*	44.65	50.06	15.69
98-23704	98-2211-2	*B. juncea/B. rapa*	42.57	48.78	27.77
98-23705	98-2213-2	*B. juncea/B. rapa*	40.74	48.92	46.42
98-23707	98-2213-3	*B. juncea/B. rapa*	43.93	50.97	15.86
98-23708	98-2214-4	*B. juncea/B. rapa*	43.31	48.21	13.48
98-23709[b]	98-2215-2	*B. juncea/B. rapa*	41.43	51.01	20.09
98-23710	98-2216-1	*B. juncea/B. rapa*	42.02	49.68	18.02
98-23711	98-2217-2	*B. juncea/B. rapa*	37.05	47.71	38.98
98-23712	98-2219-2	*B. juncea/B. rapa*	41.48	50.46	15.15
98-23714	98-2219-5	*B. juncea/B. rapa*	44.15	50.14	13.08
98-23715	98-2220-2	*B. juncea/B. rapa*	42.60	48.71	28.00
98-23716	98-2222-4	*B. juncea/B. rapa*	36.29	48.62	27.14
98-23717	98-2253-1	*B. juncea/B. rapa*	44.22	49.16	18.12
98-23718	98-2253-5	*B. juncea/B. rapa*	47.10	46.18	14.93
		Mean[c]	42.20	49.15	22.84
		SE[c]	2.52	1.39	9.28

Table 5.2 Relative proportions of nutritionally important fatty acids (in percent) in seed oil of 21 BC$_2$F$_3$ *B. juncea/B. rapa* interspecific hybrid lines and *B. juncea* (94-644 and 95-1600) and *B. rapa* (97-12929) parental lines. a = field checks (98-23699, 98-23706, and 98-23713), b = bulk seed, c = statistical parameter calculated for 21 BC$_2$F$_3$ *B. juncea/B. rapa* interspecific hybrid lines. SE = standard error.

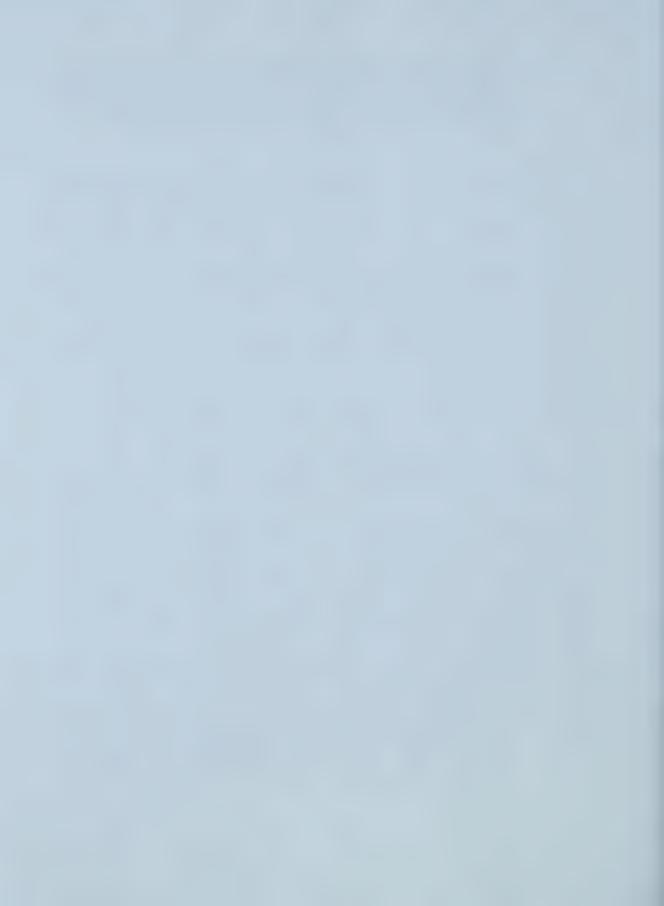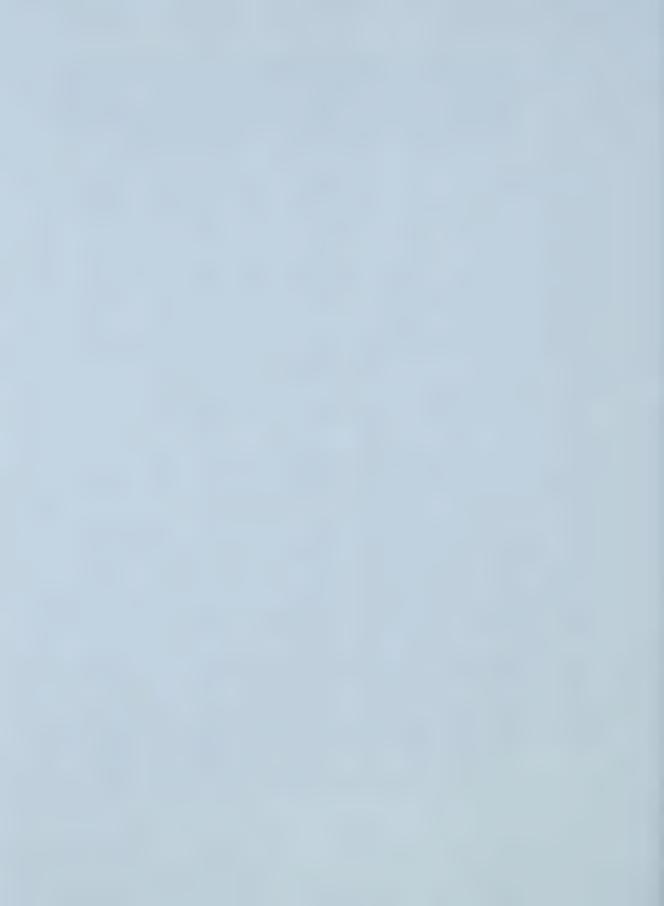| Field line	Reference	Species	Palmitic (%)	Stearic (%)	Oleic (%)	Linoleic (%)	Linolenic (%)	Erucic (%)	Saturates (%)
Parent[a]	97-12929	*B. rapa*	3.67	1.64	54.33	25.90	11.73	0.05	6.11
Parent	94-644	*B. juncea*	1.96	0.92	9.93	18.81	11.66	44.68	4.98
Parent	95-1600	*B. juncea*	4.14	2.13	26.49	29.17	9.83	16.02	7.77
98-23695	98-2205-1	*B. juncea/B. rapa*	3.84	1.60	49.88	28.81	12.77	0.11	6.28
98-23696	98-2205-3	*B. juncea/B. rapa*	3.70	1.80	55.41	24.33	12.09	0.05	6.35
98-23697	98-2205-4	*B. juncea/B. rapa*	3.65	1.41	57.04	23.71	11.35	0.12	5.85
98-23698	98-2207-1	*B. juncea/B. rapa*	3.78	1.76	54.54	25.89	11.31	0.04	6.36
98-23700	98-2209-1	*B. juncea/B. rapa*	3.69	1.27	54.68	25.70	12.04	0.07	5.73
98-23701	98-2209-5	*B. juncea/B. rapa*	3.72	1.42	54.19	25.76	12.15	0.05	5.89
98-23702	98-2210-2	*B. juncea/B. rapa*	3.94	1.59	53.82	25.85	11.71	0.23	6.34
98-23703	98-2210-3	*B. juncea/B. rapa*	3.30	1.71	57.47	23.86	10.89	0.05	5.81
98-23704	98-2211-2	*B. juncea/B. rapa*	3.30	1.28	53.95	26.21	12.65	0.04	5.30
98-23705	98-2213-2	*B. juncea/B. rapa*	3.71	1.75	50.69	28.84	12.31	0.04	6.26
98-23707	98-2213-3	*B. juncea/B. rapa*	3.68	1.52	55.12	25.02	12.13	0.05	5.94
98-23708	98-2214-4	*B. juncea/B. rapa*	3.50	2.08	54.15	23.46	13.68	0.06	6.54
98-23709[b]	98-2215-2	*B. juncea/B. rapa*	3.32	1.57	55.51	23.50	13.41	0.08	5.63
98-23710	98-2216-1	*B. juncea/B. rapa*	3.51	1.54	57.35	23.14	11.77	0.05	5.82
98-23711	98-2217-2	*B. juncea/B. rapa*	3.98	1.66	50.55	28.01	12.57	0.08	6.57
98-23712	98-2219-2	*B. juncea/B. rapa*	3.29	1.67	56.64	23.88	11.67	0.06	5.79
98-23714	98-2219-5	*B. juncea/B. rapa*	3.66	1.52	54.87	25.30	12.15	0.04	5.91
98-23715	98-2220-2	*B. juncea/B. rapa*	3.22	1.97	60.07	21.77	10.16	0.01	6.07
98-23716	98-2222-4	*B. juncea/B. rapa*	3.74	2.01	53.41	26.34	11.32	0.06	6.70
98-23717	98-2253-1	*B. juncea/B. rapa*	3.19	1.61	58.00	23.20	11.36	0.03	5.53
98-23718	98-2253-5	*B. juncea/B. rapa*	3.36	1.97	59.71	20.51	11.85	0.02	6.16
		Mean[c]	3.58	1.65	55.10	24.91	11.97	0.06	6.04
		SE[c]	0.24	0.23	2.71	2.13	0.80	0.05	0.37

Table 5.3 Seedcoat color, days-to-first flower, and days-to-maturity results reported as average values for 21 BC_2F_3 *B. juncea/B. rapa* interspecific hybrid lines and *B. juncea* (94-644 and 95-1600) and *B. rapa* (97-12929) parental lines. a = field checks (98-23699, 98-23706, and 98-23713), b = bulk seed sample, c = statistical parameter calculated for 21 BC_2F_3 *B. juncea/B. rapa* interspecific hybrid lines, d = averaged field check values, n/a = not applicable, NT = not tested, SE = standard error.

Field line	Reference	Species	Seedcoat color	Days-to-first flower	Days-to-maturity
Parent[a]	97-12929	*B. rapa*	yellow	45.67[d]	100[d]
Parent	94-644	*B. juncea*	brown	NT	NT
Parent	95-1600	*B. juncea*	brown	NT	NT
98-23695	98-2205-1	*B. juncea/B. rapa*	yellow	48	106
98-23696	98-2205-3	*B. juncea/B. rapa*	yellow	50	106
98-23697	98-2205-4	*B. juncea/B. rapa*	yellow	45	101
98-23698	98-2207-1	*B. juncea/B. rapa*	yellow	49	108
98-23700	98-2209-1	*B. juncea/B. rapa*	yellow	46	104
98-23701	98-2209-5	*B. juncea/B. rapa*	yellow	47	102
98-23702	98-2210-2	*B. juncea/B. rapa*	yellow	45	102
98-23703	98-2210-3	*B. juncea/B. rapa*	yellow	45	101
98-23704	98-2211-2	*B. juncea/B. rapa*	yellow	48	102
98-23705	98-2213-2	*B. juncea/B. rapa*	yellow	44	97
98-23707	98-2213-3	*B. juncea/B. rapa*	yellow	48	98
98-23708	98-2214-4	*B. juncea/B. rapa*	yellow	50	102
98-23709[b]	98-2215-2	*B. juncea/B. rapa*	yellow	47	100
98-23710	98-2216-1	*B. juncea/B. rapa*	yellow	45	102
98-23711	98-2217-2	*B. juncea/B. rapa*	yellow	50	101
98-23712	98-2219-2	*B. juncea/B. rapa*	yellow	45	96
98-23714	98-2219-5	*B. juncea/B. rapa*	yellow	48	100
98-23715	98-2220-2	*B. juncea/B. rapa*	yellow	49	100
98-23716	98-2222-4	*B. juncea/B. rapa*	yellow	50	99
98-23717	98-2253-1	*B. juncea/B. rapa*	yellow	45	107
98-23718	98-2253-5	*B. juncea/B. rapa*	yellow	51	95
		Mean[c]	n/a	47.38	101.38
		SE[c]	n/a	2.18	3.46

5.4 Discussion

The oil content measurements obtained by NIR-WINISI analysis were presented as a percentage on a whole seed, dry basis (Table 5.1). The oil content of the 21 BC_2F_3 *B. juncea/B. rapa* interspecific hybrid lines grown in the field observation trial ranged from 36 to 47%, with an average of approximately 42%. These values are consistent with the range commonly observed for oilseed *Brassica* species of 35 to 44% (Downey and Rimmer 1993), or 40 to 50% (Rosa 1999). The oil content of the *B. rapa* parent, estimated at 42%, equaled the calculated average obtained for the 21 BC_2F_3 *B. juncea/B. rapa* interspecific hybrid lines. The amount of seed oil present in *B. rapa* is intermediary to *B. napus* (high) and *B. juncea* (low) on a between species basis. Seed from *B. juncea* line 95-1600, used as the female parent in the original interspecific cross, was not available in sufficient amounts to determine oil content by NIR-WINISI analysis. Instead, *B. juncea* breeding line 94-644, used as a formative parent in the production of line 95-1600, provided an indication of the oil content of the *B. juncea* parent in the initial cross of *B. juncea* x *B. rapa* (See Fig 2.1). From three separate readings, breeding line 94-644 was observed to contain an average oil content of 35%. Although the 7 percentage point difference between the *B. juncea* and *B. rapa* parents could not be shown to be statistically significant, the similar oil contents of 42% in the seed of the *B. rapa* parental line 97-12929 and the 21 BC_2F_3 *B. juncea/B. rapa* interspecific hybrid lines (on average) allude to the preferential influence of the *B. rapa* parent in oil content inheritance. The two backcross generations to *B. rapa* may have contributed to the similarity of the overall oil content in the *B. rapa* parental and *B. juncea/B. rapa* interspecific hybrid lines.

Downey (1983) reported the average *B. rapa* fatty acid profile to contain oleic, linoleic and linolenic acid levels of 55, 25, and 13%. The average values for these important fatty acids were 55.10, 24.91, and 11.97% in the 21 BC_2F_3 *B. juncea/B. rapa* interspecific hybrid lines and 54.33, 25.90, and 11.73% in *B. rapa* parental line 97-12929, respectively (Table 5.2). The nearly identical values between these three *B. rapa* fatty acid profiles suggests a strong influence of the *B. rapa* parent in respect to fatty acid expression in the BC_2F_3 *B. juncea/B. rapa* interspecific hybrid lines. A possible explanation for these similarities could be the two backcross generations to the *B. rapa* parent that followed the original interspecific cross. The preferential pairing of the *B. rapa* A-genome chromosomes of the backcross parent to those in the interspecific hybrid lines would be expected to increase the proportion of *B. rapa* DNA within the interspecific hybrid lines with each successive backcross event. The regular formation of 10 bivalent chromosome associations during meiosis supports this hypothesis (See Chapter 3). The approximate proportions of oleic (10 and 26%) and linoleic (19 and 29%) acid levels in the *B. juncea* parents (94-644 and 95-1600), when compared to these parameters in the typical *B. rapa* fatty acid profile reported by Downey (1983), lend further support to the disproportionate genetic influence of the *B. rapa* parent on trait expression in the BC_2F_3 *B. juncea/B. rapa* interspecific hybrid plants.

The presence of erucic acid levels approaching the lower limit (i. e. zero) of the detection apparatus in the BC_2F_3 *B. juncea/B. rapa* interspecific hybrid lines (0.06%) and *B. rapa* parental line 97-12929 (0.05%), compared to *B. juncea* parental lines 94-644 (44.68%) and 95-1600 (16.02%), also illustrates the significant presence of *B. rapa* genetic material in the interspecific hybrid plants (Table 5.2). The average erucic acid

level of 0.06% in the BC_2F_3 *B. juncea/B. rapa* interspecific hybrid lines is much lower than the 2% maximum of the Seeds Act (Seeds Regulations of the Canadian Seeds Act 1989) and the less than 0.5% requirement of the WCC/RRC criteria for canola quality cultivars (WCC/RRC Procedures and Appendices 1997).
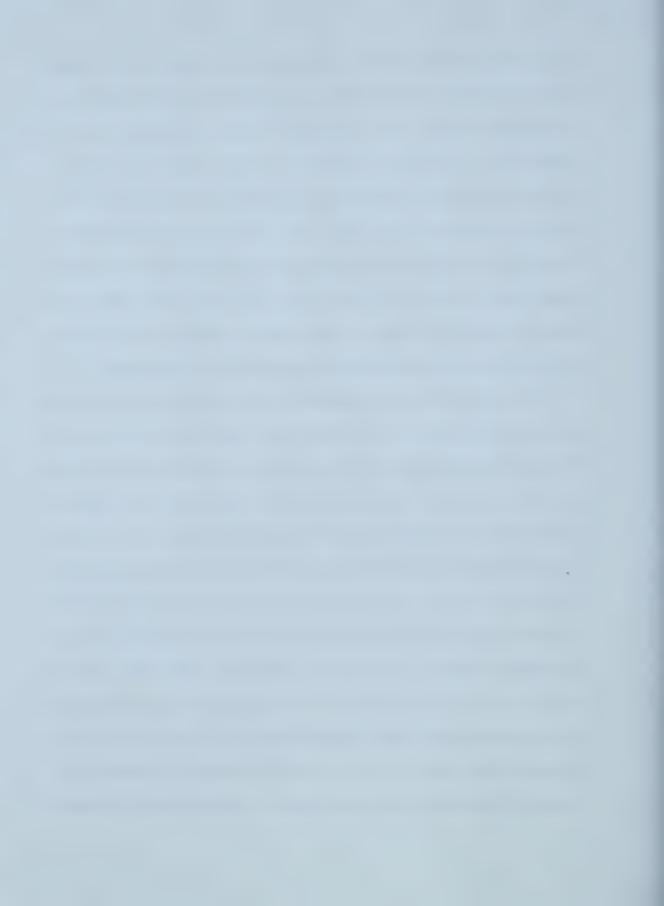
A saturated fatty acid level of 7% is expected in the seed oil of *B. napus*, while *B. rapa* levels are usually 2% less (Downey and Rimmer 1993). The saturated fat levels (i. e. the sum total of palmitic (C16:0), stearic (C18:0), arachidonic (C20:0) and behenoic (C22:0) acid) in the 21 BC_2F_3 *B. juncea/B. rapa* interspecific hybrid lines (6.01% with a standard deviation of 0.37%) and the *B. rapa* parental line 97-12929 (6.11%) were more similar to each other than the levels observed in the *B. juncea* parental lines 94-644 (4.98%) and 95-1600 (7.77%) (Table 5.2). The similarity of the saturated fatty acid values for the interspecific hybrid and *B. rapa* parental lines also supports the hypothetical reconstitution of the *B. rapa* diploid A-genome in the BC_2F_3 *B. juncea/B. rapa* interspecific hybrid lines and the strong influence of the *B. rapa* parent on trait inheritance.

Röbbelen and Thies (1980b) estimated the protein content of the dried oil-free meal for the *Brassica* species at 40%. Newkirk *et al.* (1997) have reported a range of 38 to 46%, averaged across the three major oilseed crops, along with specific estimates for *B. juncea* (45.9%), *B. napus* (44.6%), and *B. rapa* (43.1%). The average amount of protein present in the air-dried oil-free meal of the 21 BC_2F_3 *B. juncea/B. rapa* interspecific hybrid lines grown in the 1999 field observation trial was approximately 49.2% (Table 5.1). The range of individual plant readings comprising this average value (46.18 to 51.67%), as well as the results for *B. juncea* parent 94-644 (46.06%) and

B. rapa parent 97-12929 (48.37%), were greater than the protein content estimates reported by Röbbelen and Thies (1980b) and Newkirk *et al.* (1997). These elevated protein levels could have either been controlled directly at the molecular level as a heritable trait or resulted from a genotype by environment interaction. Controlled-environment testing in a greenhouse or growth cabinet, or replicated field trials at multiple sites over several years, could be used to determine the inheritance of protein content in the 21 BC_2F_3 *B. juncea/B. rapa* interspecific hybrid lines. The reversal of ranking observed between the *B. juncea* and *B. rapa* protein content values for the parental lines used in the current study and the species estimates given by Newkirk *et al.* (1997) can be attributed to the use of different genotypes in the two experiments.

The current standard for glucosinolate content in edible *Brassica* oilseed crops grown in Canada is 30 micromoles per gram or less of aliphatic glucosinolates in the oil-free meal (Seeds Regulations of the Canadian Seeds Act 1989). With extensive breeding and selection for decreased glucosinolate content during the 1980s and 1990s, the majority of the canola varieties currently being grown, developed or released actually contain a total aliphatic glucosinolate (i. e. total alkenyl plus total indoyl glucosinolates) content much lower than 10 micromoles per gram. In 1997, the Western Canadian Canola/Rapeseed Recommending Committee (WCC/RRC) established new criteria for glucosinolate evaluation in candidate cultivars. Under the WCC/RRC criteria, seed must either contain no more than 12 micromoles of total glucosinolates per gram of whole seed at 8.5% moisture content or contain total glucosinolate levels no greater than those of the designated check, whichever is higher (WCC/RRC Procedures and Appendices 1997). Analysis of NIR-WINISI results provides a quick and efficient assay able to measure

total glucosinolate content. In Europe, as in western Canada, the total glucosinolate value is currently being used to report glucosinolate content.

Total glucosinolate content in the oil-free meal of individual plants from the 21 BC_2F_3 *B. juncea/B. rapa* interspecific hybrid lines and *B. rapa* parental line 97-12929 were measured at 8.5% moisture and recorded in micromoles per gram (Table 5.1). For the NIR-WINISI-derived total glucosinolate content of the 21 BC_2F_3 *B. juncea/B. rapa* interspecific hybrid lines (on a by line basis), the average was approximately 22.84 micromoles per gram (standard error = 9.28) and there were no values meeting the minimum WCC/RRC requirement of 12 micromoles or less (WCC/RRC Procedures and Appendices 1997). The range of values from 13.08 to 46.42 micromoles per gram indicate that most, if not all, of the 21 *B. juncea/B. rapa* interspecific hybrid lines are still segregating for the glucosinolate trait. The glucosinolate contents of BC_2F_3 lines 98-2209-1 (13.21 micromoles per gram), 98-2214-4 (13.48 micromoles per gram), and 98-2219-5 (13.08 micromoles per gram), more than one standard deviation away from the mean, indicate the presence of transgressive segregation for the glucosinolate trait in the interspecific hybrid lines. A total glucosinolate content of 99.41 micromoles per gram was obtained for *B. juncea* parental line 94-644. Compared to the glucosinolate content of BC_2F_3 line 98-2213-2 (i. e. 46.42 micromoles per gram), which represents the highest value observed in the 21 *B. juncea/B. rapa* interspecific hybrid lines, the reading for the *B. juncea* parent was greater than twice this amount. Since the *B. juncea* parent was not grown in the 1999 field observation trial (due to limited seed), a feasible explanation for the 99.41 value is the influence of genotype x environment interactions on glucosinolate content. The influence of the environment has been verified as a major source of variance

on quantitative trait expression. Both the magnitude and range of glucosinolate content expression could have been influenced by one or more biotic factor(s), such as temperature, humidity or light intensity, or abiotic inputs contributing to plant growth and development (*e.g.* nitrogen availability, sulfur content of soil). The relatively high glucosinolate content of the *B. juncea* parent (i. e. 99.41), contrasted by the similar average glucosinolate content of the interspecific hybrid lines (i. e. 22.84) and the *B. rapa* parent (i. e. 20.91), suggests the strong influence of the *B. rapa* parent on glucosinolate inheritance.

B. rapa requires 85 to 90 days from seeding to reach maturity, and matures approximately 10 days earlier than *B. juncea* and *B. napus*. A shorter period to first flowering and 2 or 3 days added to the days-to-maturity of the *B. rapa* parent would be expected to increase the yield potential of *B. rapa* varieties, which has reached a plateau over the past 15 years (Stringam personal communication). The introgression of these traits into the *B. rapa* parental lines from the *B. juncea* parent, using *B. juncea/B. rapa* interspecific hybrid lines as a medium, followed by reconstitution of the *B. rapa* genome and phenotype was attempted. The three 6-meter rows of *B. rapa* parental line 97-12929 in the field observation trial reached first-flower at 45, 45, and 47 days (average = 45.67 days) after sowing and reached physiological maturity after 97, 101, and 102 days (average = 100 days) (Table 5.3). The average number of days-to-first flower for the 21 BC_2F_3 *B. juncea/B. rapa* interspecific hybrid lines was approximately 47 ± 2 days, with 6 lines producing flowers after 45 days and one line having a days-to-first flower value of 44 days. The days-to-maturity average for the interspecific hybrid lines was approximately 101 ± 3.5 days, where individual line values ranged from 95 to 107 days

(Table 5.3). A number of BC_2F_3 *B. juncea/B. rapa* interspecific hybrid lines matured 2 or more days after the 100-day average value obtained for *B. rapa* parental line 97-12929 (i. e. 102 days – 5 lines, 106 days – 2 lines, 107 days – 1 line, and 108 days – 1 line). Based on these preliminary field observation trial results, sufficient genetic variation for the days-to-first flower and days-to-maturity traits is present within the 21 BC_2F_3 *B. juncea/B. rapa* interspecific hybrid lines to allow selection for *B. rapa*-biotypes with improved days-to-first flower (i. e. 1 or 2 days less) and days-to-maturity (i. e. 2 or 3 days more), compared to *B. rapa* parental line 97-12929.

Each BC_2F_3 *B. juncea/B. rapa* interspecific line was selected for yellow seedcoat color through selection of yellow-seeded BC_2F_2 lines with yellow-seeded BC_2F_1 parents. *B. juncea* parental lines 94-644 and 95-1600 expressed an apparent non-segregating brown seedcoat color, based on only brown-seeded progeny being obtained upon self-pollination (Table 5.3). In the absence of selection for yellow seed, segregation for the seedcoat color trait was observed in all of the *B. juncea/B. rapa* interspecific hybrid generations, with brown and yellow-brown seedcoats both being dominant to yellow.

B. rapa parental lines 97-12917 and 97-12929 were originally selected as self-compatible (dominant trait) siblings from an open-pollinated self-incompatible population derived from the interspecific cross *B. rapa* x [*B. napus* x *B. napus*]. After several self-pollination generations, these self-compatible *B. rapa* biotypes displayed excellent vigor and good quality traits. Any fertility problems observed in these lines have been attributed to residual heterozygosity for chromosomal abnormalities associated with the original interspecific cross, rather than inbreeding depression, since vigor and general agronomic performance of these lines was acceptable (Stringam personal

communication). When grown in a controlled environment (i. e. Conviron growth cabinet), F_1 *B. juncea/B. rapa* interspecific hybrid plants produced malformed pods and a only few seeds per plant. BC_1F_1 *B. juncea/B. rapa* interspecific hybrid plants also displayed reduced pollen and ovule fertility and reduced seed set. These fertility problems were probably a direct result of chromosomal stress induced by the interspecific hybridization event. The greenhouse-grown BC_2F_1 and BC_2F_2, and field-grown BC_2F_3 generations produced *B. rapa* biotypes with good plant vigor and well-formed pods containing very few aborted seeds.

5.5 Conclusions

The 21 BC_2F_3 *B. juncea/B. rapa* interspecific hybrid lines expressed low erucic acid (C22:1) levels, total glucosinolate content higher than the 12-micromoles or less criterion stipulated by the Western Canadian Canola/Rapeseed Recommending Committee (WCC/RRC), average oil and protein content of approximately 42.2 ± 2.5 % and $49.2 \pm 1.4\%$, respectively, and a fatty acid profile containing the typical oleic (C18:1), linoleic (C18:2), and linolenic (C18:3) acid levels expected for *B. rapa*, as reported by Downey (1983). Yellow seedcoat color in the 21 BC_2F_3 *B. juncea/B. rapa* interspecific hybrid lines did not segregate in the BC_2F_4 progeny. The 21 BC_2F_3 *B. juncea/B. rapa* interspecific hybrid lines displayed sufficient genetic variation to facilitate selection of yellow-seeded *B. rapa*-biotypes with earlier flowering (1 or 2 days) and later maturity (2 or 3 days) than *B. rapa* parental line 97-12929 (days-to-first flower = 45.67, days-to-maturity = 100).
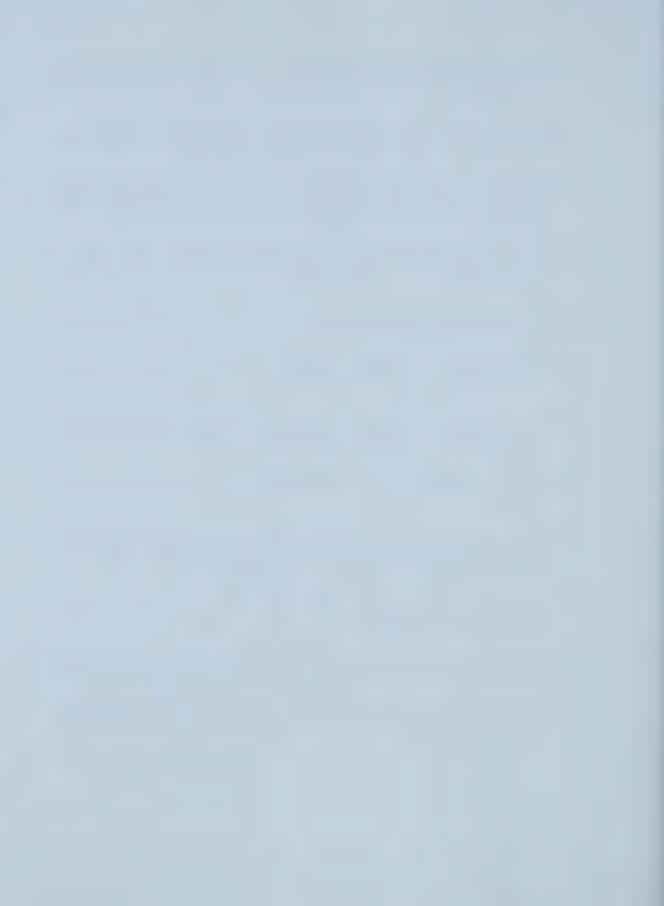
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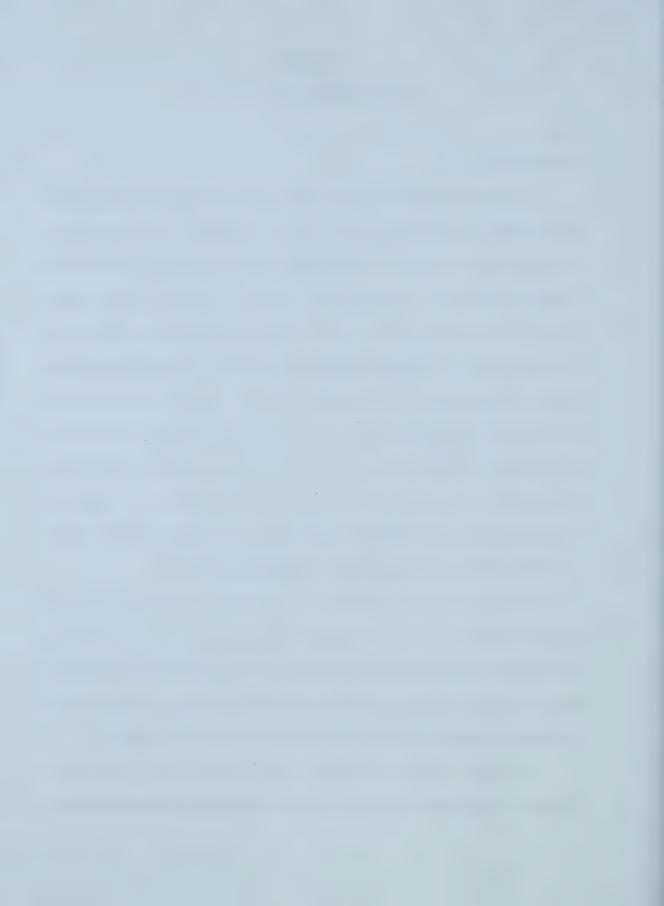
Chapter 6

General discussion and conclusions.

6.1 General discussion

The primary objective of the current study was the development of yellow-seeded, self-compatible *B. rapa* biotypes with acceptable oil and protein content, and resistance to the white rust (races 2a and 7v) and blackleg diseases of canola. *B. juncea/B. rapa* interspecific hybrids were developed through interspecific hybridization of *B. juncea* Czern. and Coss. and *B. rapa* L. to enable trait introgression between these species. Microspore culture of immature pollen grains failed to produce doubled haploid plants homozygous at all genetic loci. Trait introgression from *B. juncea* to *B. rapa* was studied for oil, protein, and glucosinolate content, fatty acid profile analysis, days-to-first flower, days-to-maturity, seedcoat color, and self-incompatibility. Cytological analysis of the *B. juncea/B. rapa* interspecific hybrid lines was used to determine the genic and chromosomal state, while RAPD-PCR marker analysis verified the presence of genetic material from both formative parents in the interspecific hybrid lines.

Following the interspecific cross *B. juncea* Czern. and Coss. x *B. rapa* L., an additional backcross to the *B. rapa* parent was performed (Fig. 2.1). Based on intermediary physical characteristics between the formative parents and/or physiological stresses commonly observed in Brassica interspecific hybrids, two of the BC_1F_1 generation plants were labeled as putative *B. juncea/B. rapa* interspecific hybrids.

Microspore culture of individual BC_1F_1, BC_1F_2, BC_2F_1, and BC_2F_2 *B. juncea/B. rapa* interspecific hybrid plants produced very few torpedo-shaped embryos,

which had the ability to produce significantly higher frequencies of regenerated plants than other embryo types (Chuong and Beversdorf 1985). The majority of viable embryoids developed into callus-like masses of undifferentiated cells that required continuous subculturing before the sporadic production of the first true-leaf and a healthy rooting system. As a result, only 1 colchicine-treated and 3 spontaneous DH *B. juncea/B. rapa* plants/lines were obtained from 1339 embryos produced by 611 separate culture events. Yields from these 4 DH_1 plants were inadequate in providing sufficient seed for the planned 2-year, two-location field trial. Therefore, 21 yellow-seeded BC_2F_2 *B. juncea/B. rapa* interspecific hybrid lines were sown in single 6-meter observation rows during the 1999 field season. Days-to-first flower, days-to-maturity, and seedcoat color were recorded and individual BC_2F_3 generation plants were selected for seed oil, protein, and glucosinolate analyses.
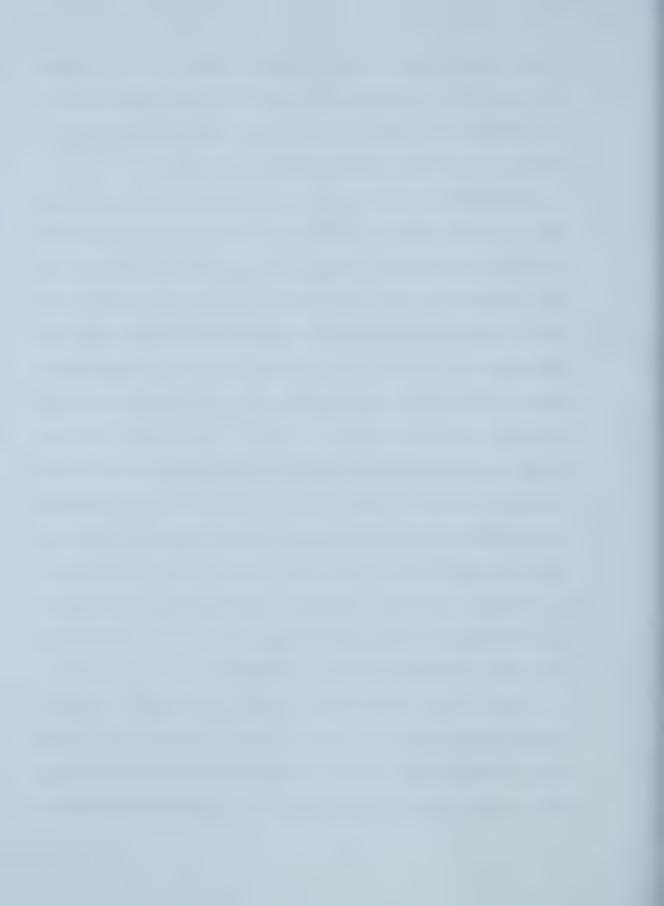
Chromosome counts in PMCs from the 21 BC_2F_2 *B. juncea/B. rapa* interspecific hybrid lines revealed the regular formation of 10 bivalent chromosome associations during meiosis. Reconstitution of the A-genome in the BC_2F_2 *B. juncea/B. rapa* interspecific hybrid lines may have occurred through genomic stabilization, which is the formation of only full complements of haploid chromosome sets during meiosis (Song *et al.* 1993). Preferential production of A-genome gametes in the F_1 interspecific hybrid, coupled with fertilization by the *B. rapa* backcross parent, could account for the 10 bivalent chromosome associations and the *B. juncea* and *B. rapa* genetic material present in the BC_2F_2 *B. juncea/B. rapa* interspecific hybrid lines. Although the previous scenario is possible, a more likely explanation for the reconstituted *B. rapa* A-genome is that the two backcross generations effectively promoted preferential pairing between homologous

B. rapa A-genome chromosomes over non-homologous *B. juncea* A-genome chromosomes. The *B. juncea* B-genome univalent chromosomes present in the F_1 interspecific hybrid were probably lost through random assortment at meiosis during the backcross and self-pollination generations that followed the initial cross.

The microspore culture technique was also expected to alleviate any chromosomal imbalances (mainly inversions or translocations), or fertility problems associated with unpaired chromosomes, through colchicine-induced or spontaneous doubling of the haploid chromosome complement of the interspecific hybrid lines (Thiagarajah *et al.* 1994). The failure of the MC technique to produce a sufficient number of DH plants/lines necessitated the use of the BC_2F_2 and BC_2F_3 generations in subsequent studies. In light of this setback, the importance of cytological evaluation in respect to its ability to determine the genic and chromosomal state of the *B. juncea/B. rapa* interspecific hybrid lines increased dramatically. Additional bivalent or univalent chromosomes present in a genetic background following interspecific hybridization may impart or carry the desired or newly introgressed trait. Avoiding additional or unpaired trait-carrying chromosomes in Brassica interspecific hybrids developed for trait introgression is important, since Roy (1984) found that through random assortment and segregation of chromosomes during meiosis, *B. juncea*-type complete blackleg disease resistance was eliminated from *B. napus/B. juncea* interspecific hybrid chromosome addition lines.
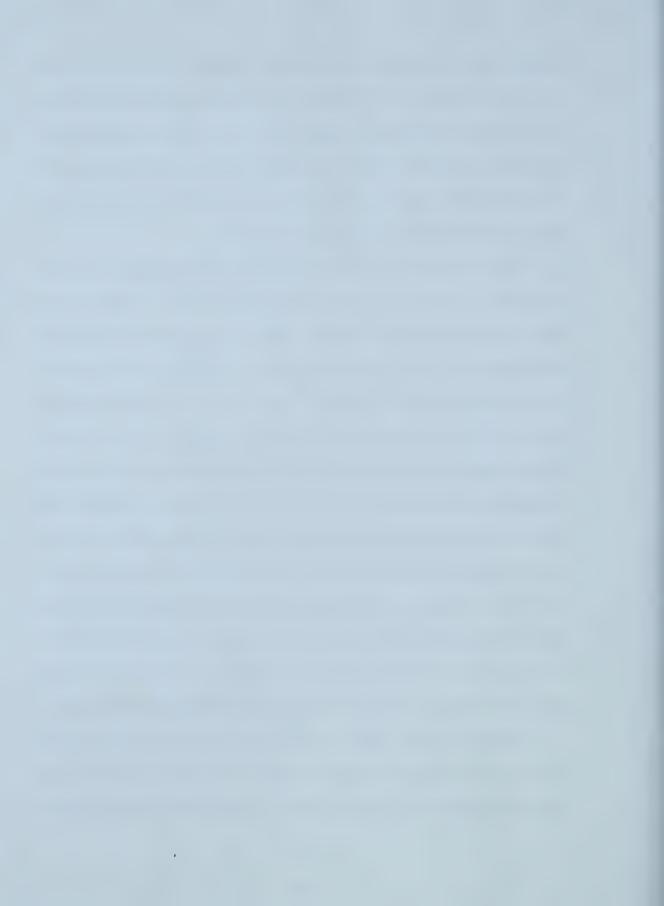
White rust and blackleg disease resistance responses in the 21 BC_2F_3 *B. juncea/B. rapa* interspecific hybrid lines mimicked those obtained for the *B. rapa* parent. For blackleg disease, moderate to highly resistant responses were observed, while the *B. rapa* parent and the *B. juncea/B. rapa* interspecific hybrid lines segregated for

resistance to race 2a of *Albugo candida* and were completely susceptible to race 7v of *A. candida*. The excellent race 7v resistance in the *B. juncea* parent was not introgressed into the interspecific hybrid lines. Another cross to the *B. juncea* parent (for race 7v introgression) and selection for race 2a resistance in the BC_2F_3 generation, along with trait selection and cytological analysis, could improve the white rust disease resistance responses in the *B. juncea/B. rapa* interspecific hybrid lines.

The results obtained for oil and protein content and the individual fatty acids in the 21 BC_2F_3 *B. juncea/B. rapa* interspecific hybrid lines were very similar to those observed for the *B. rapa* parent. The nearly identical average values of the interspecific hybrid lines and the *B.* rapa parent for oleic acid (55.10 vs. 54.33%), linoleic acid (24.91 vs. 25.90%), linolenic acid (11.97 vs. 11.73%), erucic acid (0.06 vs. 0.05%), and total oil (42.20 vs. 42.17%) contents suggests a strong influence of the *B. rapa* parent in respect to fatty acid expression and oil content inheritance (on a whole seed, dry basis) in the BC_2F_3 *B. juncea/B. rapa* interspecific hybrid lines. The dissimilarity of the above readings in the *B. juncea* parental lines for the oleic acid (9.93 and 26.49%), linoleic acid (18.81 and 29.17%), linolenic acid (11.66 and 9.83%), erucic acid (44.68 and 16.02%), and total oil (35.09%; oil content not determined for second parent) contents provides additional evidence supporting the notion that the *B. rapa* A-genome was reconstituted in the BC_2F_3 *B. juncea/B. rapa* interspecific hybrid lines and that the *B. rapa* parent strongly influenced the inheritance of the individual fatty acids in the interspecific hybrid lines.

Although the average values for oil and protein content and the individual fatty acids were almost identical, the range of values in the 21 BC_2F_3 *B. juncea/B. rapa* interspecific hybrid lines for each trait contained a considerable amount of genetic
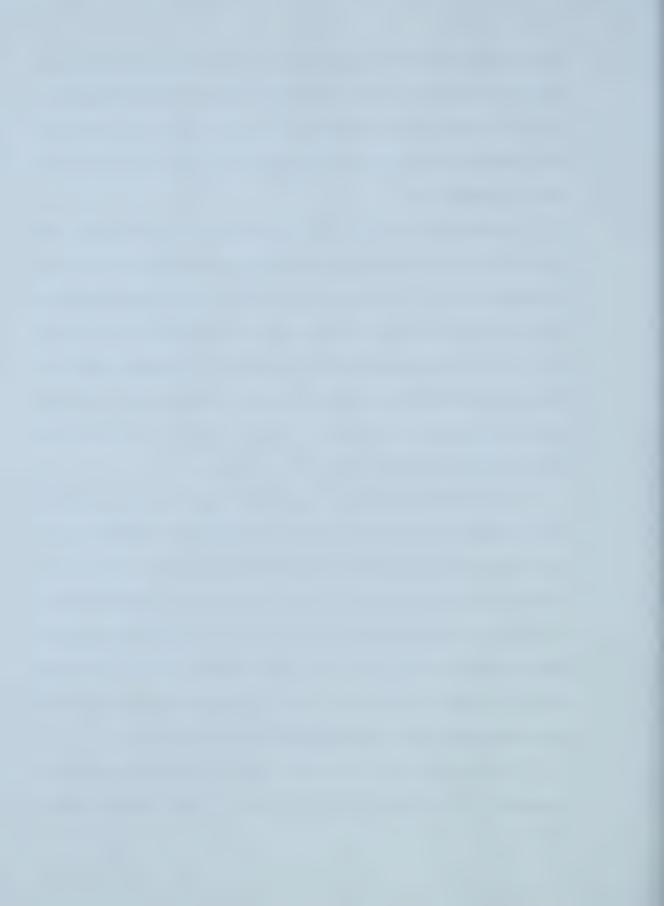
variation. Grami *et al.* (1977) reported a negative correlation between the heritability of oil and protein content in *B. napus*. As literature and production statistics suggest the absence of an upper breeding limit for oil and protein content (Downey and Rimmer 1993), simultaneous selection in future generations should result in an improved sum total for both components.

A saturated fatty acid level of 7% is expected in the seed oil of *B. napus*, while *B. rapa* levels are usually 2% less (Downey and Rimmer 1993). The similarity of the saturated fat values for the interspecific hybrid (6.01% with a standard deviation of 0.37%) and *B. rapa* parental lines (6.11%) also supports the hypothetical reconstitution of the *B. rapa* diploid A-genome in the BC_2F_3 *B. juncea/B. rapa* interspecific hybrid lines and the disproportionate influence of the *B. rapa* parent on trait inheritance. The genetic variation present between the 21 BC_2F_3 *B. juncea/B. rapa* interspecific hybrid lines should allow selection for reduced saturated fatty acid levels.

The NIR-WINISI-derived total glucosinolate content of the 21 BC_2F_3 *B. juncea/B. rapa* interspecific hybrid lines were all higher than the WCC/RRC limit of 12 micromoles of total glucosinolates per gram of whole seed at 8.5% moisture content (WCC/RRC Procedures and Appendices 1997). Although the lowest obtained value was greater than the minimal requirement, the 12 micromoles per gram value was almost within one standard deviation of the mean. As the NIR-WINISI procedure is so quick and efficient, large numbers of BC_2F_4 progeny could be screened in an attempt to reduce total glucosinolate content below the current standard observed in western Canada.

B. rapa requires 85 to 90 days from seeding to reach maturity, which is approximately 10 days earlier than *B. juncea* and *B. napus* (Stringam personal

communication). The genetic variation present in the 21 BC_2F_3 *B. juncea/B. rapa* interspecific hybrid lines for days-to-first flower and days-to-maturity might be exploited to allow 2 or 3 days more to the days-to-physiological maturity index of the *B. rapa* parent, along with a corresponding increase in yield potential. Additional field studies would be required to test this hypothesis.
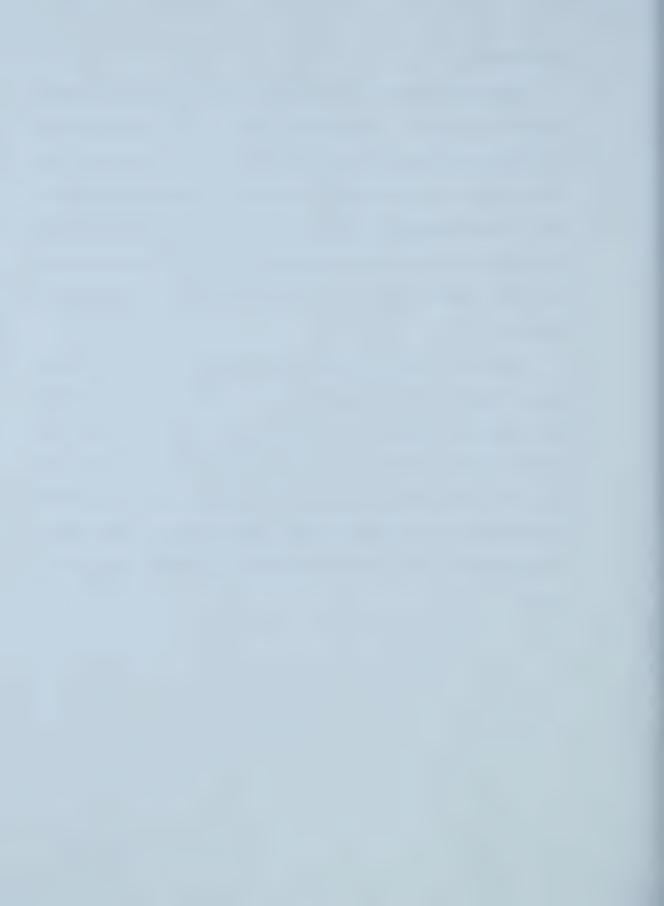
The F_7 lines used as the *B. rapa* parents in the interspecific cross between *B. juncea* and *B. rapa* expressed *B. rapa* phenotypes, excellent vigor, and good quality traits. The fertility problems observed within these lines were attributed to residual heterozygosity for chromosomal abnormalities associated with the original interspecific cross, rather than inbreeding depression, since vigor and general agronomic performance of these lines was acceptable (Stringam personal communication). Based on unrecorded seed-setting behavior and vigor observations, the 21 BC_2F_3 *B. juncea/B. rapa* interspecific hybrid lines also did not display the symptoms of inbreeding depression, i. e. a loss in vigor and reduced pollen and ovule fertility. Instead of the aborted ovules and stunted, seedless pods indicative of a self-incompatible response, there was regular development of well-formed, seed-filled pods following self-pollination. The values obtained for days-to-first flower and days-to-maturity in the *B. juncea/B. rapa* interspecific hybrid lines suggested sufficient genetic variation was present to allow future selection of yellow-seeded *B. rapa*-biotypes with earlier flowering (1 or 2 days) and later maturity (2 or 3 days) than *B. rapa* parental line 97-12929. Additional field studies would be required to test this hypothesis.
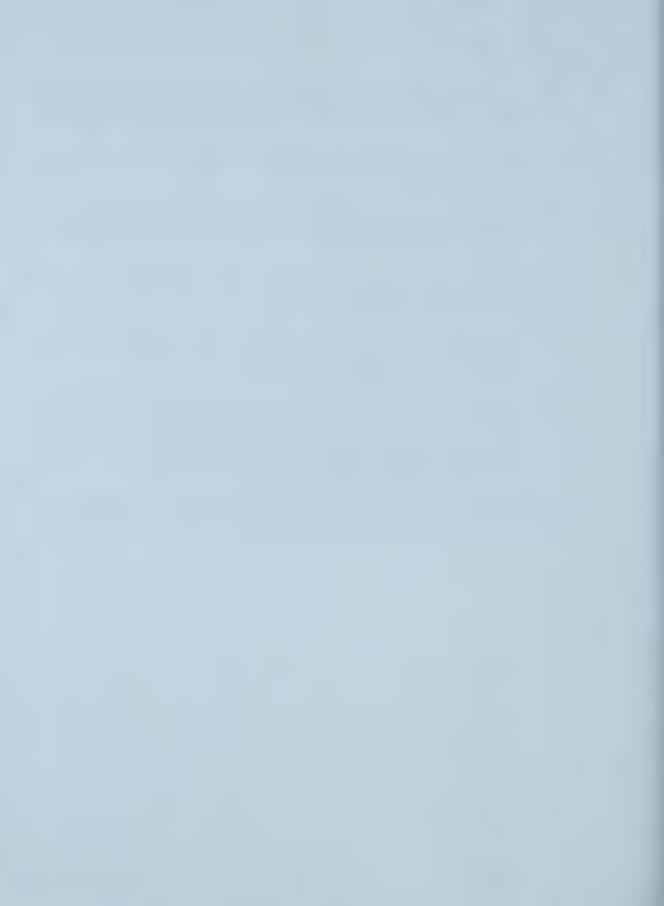
6.2 Conclusions

Introgression of agronomic, quality, and self-compatibility traits from *B. juncea* to *B. rapa* using *B. juncea/B. rapa* interspecific hybrid lines was partially successful. RAPD-PCR analysis verified the interspecific hybridization event, but the inability of the microspore culture technique to produce large quantities of haploid plantlets resulted in failure to achieve homozygosity at all genetic loci in the F_1 interspecific hybrid progeny. It is possible that *B. juncea* genetic material introgressed into the *B. rapa* A-genome may have been eliminated during the two additional backcross and self-pollination generations.

Through the introgression of genetic material from *B. juncea* to *B. rapa*, the primary objectives of the current study were nearly obtained. The BC_2F_3 *B. juncea/B. rapa* interspecific hybrid lines developed were yellow-seeded, self-compatible, contained 10 bivalent chromosome associations at diakinesis (i. e. *B. rapa*, AA, 2n=20), had acceptable oil and protein content, a typical *B. rapa* fatty acid profile, moderate to high levels of resistance to blackleg disease of canola, but were completely susceptible to race 7v of *A. candida* and segregated for resistance to race 2a of *A. candida*.

6.3 References

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Grami, B., Baker, R. J., and Stefansson, B. R. 1977. Genetics of protein and oil content in summer rape: Heritability, number of effective factors, and correlations. Can. J. Plant Sci. 57, 937-943.

Roy, N. N. 1984. Interspecific transfer of *Brassica juncea*-type high blackleg resistance to *Brassica napus*. Euphytica 33, 295-303.

Song, K., Tang, K., and Osborn, T. C. 1993. Development of synthetic *Brassica* amphidiploids by reciprocal hybridization and comparison to natural amphidiploids. Theor. Appl. Genet. 86, 811-821.

Thiagarajah, M. R., Hawkins, G. P., Stringam, G. R., Bansal, V. K., and Johnson-Flanagan, A. M. 1994. Use of doubled haploids to introgress traits between *Brassica juncea* and *Brassica napus*. (Abstr.) The methodology of plant genetic manipulation: Criteria for decision making, Eucarpia section: Genetic manipulation in plant breeding, Cork, Ireland, pp. 11-14, Sept. 1994.

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Appendices

A.1 Appendix A (from Coventry *et al.* 1988; See Section 2.6 for reference):

B_5 Wash (1L)

B_5 x 10 stock (below)	100 ml
sucrose	130 g

ddH$_2$O added to a final volume of 1 L, stirred contents until dissolved, adjusted pH between 5.8-6.0, and sterilized in an autoclave.

B_5 x 10 Stock (frozen) (1 L)

KNO$_3$	12.50 g
MgSO$_4$-7H$_2$O	1.25 g
CaCl$_2$-2H$_2$O	3.75 g
(NH4)$_2$SO$_4$	0.67 g
NaH$_2$PO$_4$-H$_2$O	0.75 g
Fe 330	0.20 g
B_5 x 10 vitamin stock (below)	50.00 ml
B_5 x 100 micronutrient stock (below)	50.00 ml
KI stock (below)	5.00 ml

ddH$_2$O added to a final volume of 1 L, stirred contents until dissolved, and stored in freezer as 100 ml samples.

B_5 x 10 Vitamin Stock (frozen) (1 L)

myoinositol	10.00 g
nicotinic acid	0.10 g
pyridoxine HCl	0.10 g
thiamine HCl	1.00 g

ddH$_2$O added to a final volume of 1 L.
Stirred contents until dissolved and stored in freezer as 100 ml samples.

B_5 x 100 Micronutrient Stock (frozen) (1L)

MnSO$_4$-H$_2$O	1.00 g
H$_3$BO$_3$	0.30 g
ZnSO$_4$-7H$_2$O	0.20 g
Na$_2$MnO$_4$-2H$_2$O	0.025 g
CuSO$_4$-5H$_2$O	0.0025 g
CoCl$_2$-6H$_2$O	0.0025 g

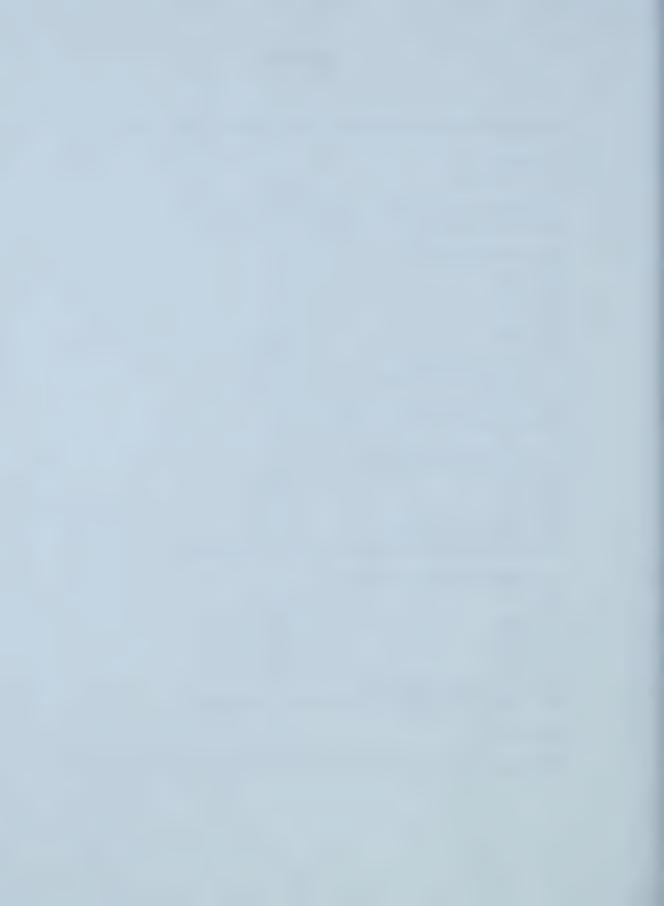ddH$_2$O added to a final volume of 1 L.
Stirred contents until dissolved and stored in freezer as 100 ml samples.

KI Stock (1L)

KI	0.83 g

ddH$_2$O added to a final volume of 1 L, stirred contents until dissolved, and refrigerated (2-5°C).

A.2 Appendix B (from Coventry *et al.* 1988; See Section 2.6 for reference):

NLN10 Culture Medium (10 L)

ddH$_2$O	1.5	L
KNO$_3$	1.25	g
MgSO$_4$-7H$_2$O	1.25	g
Ca(NO3)$_2$-4H$_2$O	5.0	g
KH$_2$PO$_4$	1.25	g
Fe 330	0.4	g
NN x 100 vitamin stock	100	ml (1 bag)
MS x 100 micronutrient stock	100	ml (1 bag)
glutathione	0.3	g
L-glutamine	8.0	g
L- serine	1.0	g
sucrose	1000	g

ddH$_2$O added to a final volume of 10 L, stirred contents until dissolved, adjusted pH between 5.8-6.0, filter sterilized, and stored in fridge (2-5°C).

NLN13 Culture Medium (10 L)
Same as NLN10 (above), except 1300 g of sucrose

NLN17 Culture Medium (10 L)
Same as NLN10 (above), except 1700 g of sucrose, no L-glutamine, 0.5 mg/l Benzyladenine

NN x 100 Vitamin Stock (frozen) (1 L)

glycine	0.2	g
myoinositol	10.0	g
nicotinic acid	0.5	g
pyridoxine HCl	0.05	g
thiamine HCl	0.05	g
folic acid	0.05	g
biotin	0.005	g

ddH$_2$O added to a final volume of 1 L, stirred contents until dissolved, and stored in freezer as 100 ml samples.
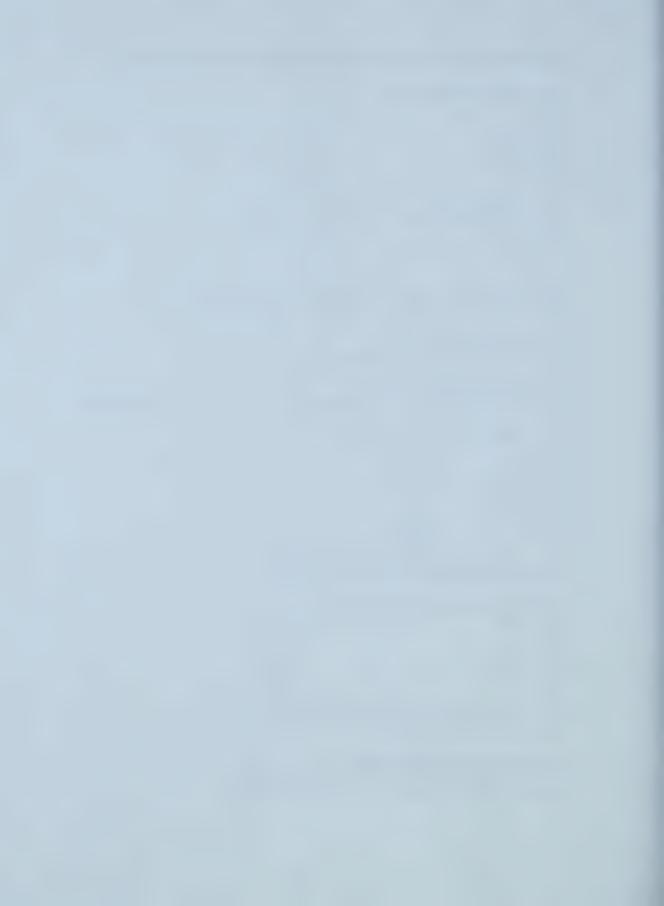
MS x 1000 micronutrient stock (frozen) (1 L)

MnSO$_4$-4H$_2$O	22.3	g
H$_3$BO$_3$	6.2	g
ZnSO$_4$-7H$_2$O	8.6	g
Na$_2$MoO$_4$-2H$_2$O	0.25	g
CuSO$_4$-5H$_2$O	0.025	g
CoCl$_2$-6H$_2$O	0.025	g

ddH$_2$O added to a final volume of 1 L, stirred contents until dissolved, and stored in freezer as 100 ml samples.

MS x 100 micronutrient stock (frozen) (1 L)
Thawed one bag (100 ml) of MS x 1000 micronutrient stock, added ddH$_2$O to 1L, and stored in freezer as 100 ml samples.
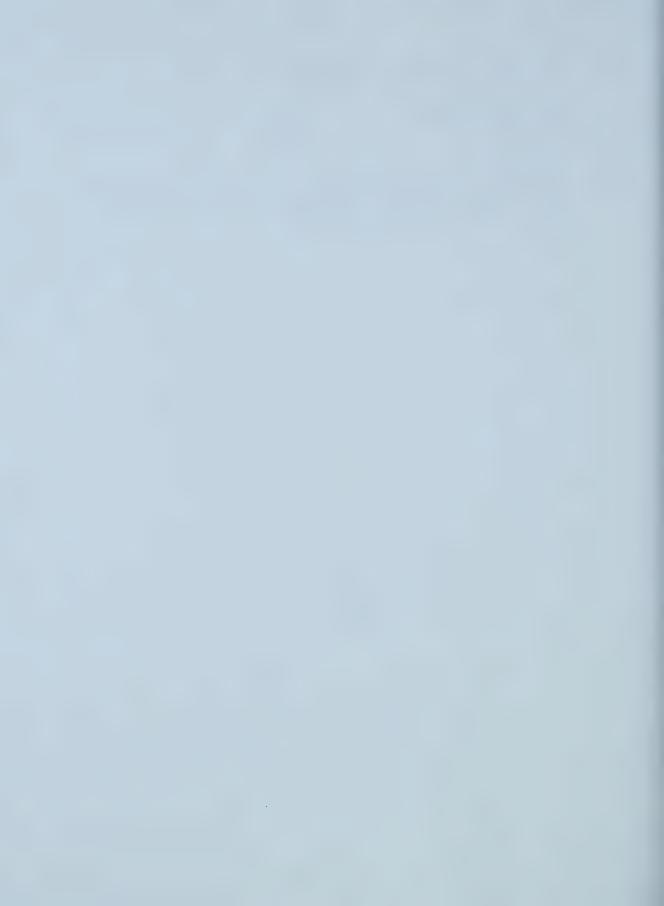
A.3 Appendix C (from Coventry *et al.* 1988):

B$_5$ Solid Culture Media (1 L)

B$_5$ x 10 stock (Appendix A)	200 ml
sucrose	20 g
GA$_3$ (0.15 mg/L)	1 ml

ddH$_2$O added to a final volume of 1 L.

Stirred contents until dissolved, adjusted pH to 5.7, sterilized in autoclave, poured 10 ml aliquots into 100 x 15mm petri plates (approximately 100 plates can be poured), allowed samples to solidify, and sealed in plastic bags until used.